VARIATION IN *PHELLINUS NOXIUS* (CORNER) G.H. CUNN.

by

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CORRIGENDA

p66, par 4, line 1. Growth rates of all isolates at each of the temperatures, $10^{o}C$, $15^{o}C$, $20^{o}C$ and $35^{o}C$ were remarkably similar.

p78, par 2, line 1-3. Hence they grew (trace) at a lower pH (2.2) than any field isolate.

p139, par 2, line 7. (Figs 6.5.4 and 6.5.5 also text 6.4.3.3)

p171, par 2, line 1. "Only three isolates (5, 12, 20) . . .

ORIGINALITY OF THESIS

Except where acknowledged this thesis is my own work.

J Ballard

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ABSTRACT

Studies of the variation in *Phellinus noxius* (Corner) G.H. Cunn. were useful for assessing options for control of severe root-rot induced by the fungus in hoop pine plantations in Queensland. Field isolates from two hosts (hoop pine and rubber) and three geographical regions (southeastern and north Queensland, and Malaysia) were examined for variation in cultural characters, in certain physiological traits (temperature- and pH-growth relationships, and extracellular enzyme production), and in pathogenicity. The limits of variation among monoarthrospore and monobasidiospore isolates derived from a single field collection were assessed to give a better appreciation of the overall variation found among field isolates. Heterokaryosis, nuclear division, and polyploidy in the pathogen were investigated as possible sources of this variation.

Cultural characters of field, monoarthrospore and monobasidiospore isolates were described in detail. They varied widely among and within field and basidiospore isolates, but were more uniform among arthrospore isolates. Field isolates, which normally were pigmented, frequently gave rise to unpigmented sectors. Three cultural types were recognized among basidiospore isolates: unpigmented appressed, intensely pigmented appressed, and an intermediate type of variable pigmentation and with a white aerial bloom. Frequent spontaneous but reversible changes occurred between the two appressed types.

The behaviour of unpigmented mycelia differed markedly from that of pigmented mycelia; the former grew faster, tended to predominate at temperatures optimal for growth, were less sensitive to changes in the pH of malt-extract agar, had a different enzyme profile (in particular, they lacked oxidase) and were non-pathogenic. Despite the within-isolate variation, differences occurred also among field isolates: those from Malaysia

grew faster, had lower pH optima, and were more virulent than those from Queensland; and those from the contexts of basidiocarps grew more slowly and were less virulent than those from tissues of the host.

The cytology of somatic mycelia, and of germinating arthrospores and basidiospores and their developing colonies were described in detail. Cells of somatic mycelia were multinucleate: apical cells at the margin of cultures contained a mean of 7.3-8.1 (range 2-26) nuclei. Arthrospores and basidiospores were mostly uninucleate, but cells of their colonies became multinucleate also, within 15-18 hr of spore germination. The nuclear condition of basidiospore isolates varied with cultural type: apical cells of both appressed types contained a mean of 6.2-9.1 nuclei, and those of the intermediate type, 15.7-16.0 nuclei. Extensive nuclear migration was apparent in all mycelia examined.

The cytology of nuclear division in somatic mycelia was described in detail also. Attempts to stain nuclei in subhymenia, basidia, and attached basidiospores in fructifications were unsuccessful: The hymenium was coated with a mucilage and the hyphae in the subhymenium were agglutinated, and these materials probably impeded the penetration of one or more of the chemicals used in the staining process. Nuclear division in somatic mycelia was asynchronous and amitotic. Daughter nuclei from the same parent often appeared to differ in chromatin content.

Heteroploidy was demonstrated in somatic mycelia by Feulgen-DNA microspectrophotometry. Nuclear volume proved also to be a good indicator of nuclear ploidy, and was used in studies in spores and monosporous mycelia. Nuclei in apical cells of somatic mycelia varied in ploidy from haploid to octaploid, but were predominantly diploid. Arthrospores perpetuated this condition. The ploidy of nuclei in mycelia of basidiospore isolates varied with cultural type: nuclei in mycelia of both appressed types were predominantly haploid, and those of the intermediate type were predominantly diploid.

A model (based on the occurrence of heteroploidy, and on cytological observations on nuclear division and migration) was proposed for nuclear mechanisms which appeared to operate in somatic mycelia. The model could account for much of the within-isolate variation observed in the fungus.

The pattern of sexual incompatibility operating in the fungus was not clearly established. Considerable difficulty was encountered in interpreting interactions of paired monobasidiospore mycelia, and additionally, most monospore isolates had unique "patterns of interactions" with other isolates. Appressed cultural types are probably heterokaryotic with haploid nuclei, and nuclei of the intermediate cultural type are probably heterozygous diploids; however, evidence from several other studies in the project suggest that none of the cultural types is homothallic. The difficulties encountered may be due to the complexities of diploid-haploid and heterokaryotic haploid-heterokaryotic haploid crosses. Since 26% of the pairings were markedly interactive and probably non-compatible, there is some evidence for tetrapolar incompatibility.

Isolates from the one geographical region are genetically more alike than those from different regions, but genetic dissimilarities occur also within the one region. One of the Malaysian isolates appeared to be genetically isolated from a north Queensland isolate suggesting that there are at least two intersterile groups among populations of the fungus from these two regions.

Further investigations of physiological traits and pathogenicity among field isolates from additional host and geographical sources might reveal local adaptations by *P. noxius*; however a more complete understanding of the nuclear mechanisms for variation is considered more important at this juncture. Before the various options for control of root-rot can be assessed, the roles of basidiospores and arthrospores in the life and disease cycles of the pathogen require elucidation also.

CHAPTER 1

GENERAL INTRODUCTION

Phellinus noxius (Corner) G.H.Cunn. (Aphyllophorales: Hymenochaetaceae) is indigenous to rainforests throughout the tropics (Appendix 1). The fungus has been reported as a pathogen on herbaceous plants, shrubs and trees from almost 70 genera in 33 families of Gymnospermae and both classes (Monocotyledones and Dicotyledones) of the Angiospermae (Appendix 2; hitherto unpublished accessions of the Queensland Department of Forestry are also included in this appendix).

Diseases reported to be incited by *P. noxius* include stem-rot of oil palm (*Elaeis guineënsis* Jacq.: Corner, 1932; Thompson, 1937; Anon. 1965), branch-dieback of rubber (*Hevea brasiliensis* Muell. Arg.: Altson, 1949; Newsam, 1966; Rao, 1970), and debilitative or lethal root-rot of a wide range of hosts (Corner, 1932; Browne, 1968; Fidalgo, 1968). Most reports of root-rot come from natural stands or plantations, but the fungus has also caused loss of seedlings in nurseries (Ramakrishnan and Radhakrishnan, 1964). *Phellinus noxius* has also been associated with decay of fallen logs (Beeley, 1939; Fox, 1965), and of timber after conversion (Singh, 1966).

The fungus has economic significance on many of its hosts. It appears to be the most important root pathogen of cocoa (*Theobroma cacao* L.), coffee (*Coffea arabica* L.), and tea (*Camellia sinensis* L.) (Coleman, 1932; Gandrup, 1932; Van der Goot, 1937; Anon., 1954; Smith, 1955; Shaw, 1963; Thrower, 1965). On rubber, it is the principal root pathogen in southern India (Riggenbach, 1966), but ranks third in importance behind *Rigidoporus lignosus* (Klotzsch) Imazeki and *Ganoderma pseudoferreum* (Wakef.) van Overeem et Steinm. in Indonesia and Malaysia (Dijkman, 1951; Fox, 1966;

Taysum, 1966). Branch-dieback of rubber incited by *P. noxius* has been described as "severe" (Rao, 1970). The widely reported stem-rot of oil palm, however, appears to be of little economic importance (Turner, 1967, 1971). The impact of the fungus on each of the above hosts may be modified by, *inter alia*, local site and host factors (Tempany, 1934; Thompson, 1937; Fernando, 1940; Sarmah, 1957; Thrower, 1965), and by cultural practices (Berwick, 1949; Young, 1952; Hutchinson, 1961; Fox, 1965).

Phellinus noxius incites root-rot on many tree species of value to forestry, but there are few published assessments of losses. The fungus has caused 10 per cent mortality in plantations of *Swietenia macrophylla* King in Fiji (Browne, 1968), and 50 per cent mortality in a three year old plantation of *Pinus merkusii* Jung & de Vriese in Indonesia (Dr I.A.S. Gibson, pers.comm.).

The fungus is reported as the most widespread and destructive basidiomycete root pathogen in plantations of indigenous and exotic conifers in Queensland (Bolland, 1978). Notable losses have occurred in plantations of hoop pine (*Araucaria cunninghamii* Ait.); the recorded distribution of *P. noxius* in these plantations is shown in Appendix 3. The fungus has killed trees of all vigour classes in plantations aged two to 49 years (Bolland, 1976). In first-rotation stands, up to 6 per cent mortality over whole compartments was recorded by age seven years; and in one 35 year old stand on the Atherton Tableland, 3.3 per cent of trees had recognizable symptoms of infection by *P. noxius* on their butts. Over 17 per cent mortality was recorded by age seven years in a second-rotation stand with a previous history of the disease. While control of the fungus is desirable in these plantations, methods which have been used elsewhere, in rubber plantations for example (Wastie, 1975), are either too costly or inappropriate for pine plantations.

Some appreciation of the variability of a pathogen would be useful

when assessing the various methods of control which might be used. Much of this appreciation can be gained from laboratory studies. It is surprising that a review of the literature published on *P. noxius* revealed that this important pathogen has rarely been studied in the laboratory: Riggenbach (1958) found the fungus was heterotrophic for thiamine; Sehgal *et al.* (1966) examined the temperature-growth relations of a single isolate; Rao (1970) reported on the temperature, pH, moisture, and broad nutritional requirements for basidiospore germination; Bakshi *et al.* (1970) described the cultural characters of a single isolate; and Cowan *et al.* (1973) identified several volatile metabolites of the fungus. The ability of *P. noxius* to colonize a wide range of habitats and hosts suggests great variation within and among isolates in, *inter alia,* physiology and pathogenicity, similar to that reported in other important basidiomycete root pathogens with wide distributions and host ranges, e.g., *Armillariella mellea* (Vahl.) Karst. and *Heterobasidion annosum* (Fr.) Bref. Studies on variation in the physiology and pathogenicity of *P. noxius* form part of the present project.

Further studies on the cultural characters of *P. noxius* will assist identification. Such studies may also reveal an association between morphological type and genotypic variation expressed as differences in physiology and pathogenicity. Such an association has been shown in *Phellinus tremulae* (Bond.) Bond. & Borisov, and in *Polyporus tomentosus* Fr. The "Bleaching" and "Staining" cultural types of *P. tremulae* differ in their tolerance of contaminating fungi in laboratory culture and have their own characteristic enzyme profiles (Hiorth, 1965), and differ in growth rate on nutrient agar (Niemelä, 1977a). The pathogenicity of isolates of *P. tomentosus* is correlated with the intensity of mycelial pigmentation in culture (Whitney & Bohaychuk, 1977).

As indicated previously, knowledge of the physiology of *P. noxius* is fragmentary. Elementary studies on the temperature-growth and pH-growth relations are necessary to establish optimal conditions for the isolation and growth of the fungus in laboratory culture, and they might reveal also physiological adaptation in the fungus. For example, Courtois (1974) and de Azevedo and Moniz (1974) found two ecotypes with different pH optima among isolates of *H. annosum* derived from hosts growing on calcareous soils and on soils deficient in lime. Recent studies on extracellular enzymes produced by Hymenomycetes and other fungi in laboratory culture have shown that differences occur between species which are related taxonomically, or which compete for similar substrates (Taylor, 1974, 1977; Hankin & Anagnostakis, 1975; Raper & Kaye, 1978). Variation in enzyme production has been recorded among isolates of the one hymenomycete species (e.g. Federov & Staichenko, 1974; Taylor, 1974), and even within the one isolate (Hiorth, 1965; Raper & Kaye, 1978). Wide enzyme variability might indicate a similar variability in aggressiveness and pathogenicity. Hence, assays for extracellular enzymes might be useful in revealing variation in *P. noxius*.

Studies on cultural features, physiology, and pathogenicity of isolates from a range of host, site, and geographical sources may indicate possible variation in plant pathogenic fungi. However, the key to understanding variability in such fungi lies in their genetic systems. Genetic diversity in plant pathogens is generated by a variety of extranuclear and nuclear mechanisms (Raper, 1966; Webster, 1974; Burnett, 1975). Until recently, little was known of genetic variation in hymenomycete root pathogens. The studies of Tommerup and Broadbent (1975), Korhonen (1978a) and Peabody *et al.* (1978) on *A. mellea,* and of Korhonen (1978b) on *H. annosum*, have contributed significantly to an understanding

of the wide variation existing in these fungi.

Little attention has been given to genetic variation in the Hymenochaetaceae, although some aspects of cytology and sexual and vegetative incompatibility have been investigated in *Phellinus gilvus* (Schw.ex Fr.) Pat., *Phellinus igniarius* (L. ex Fr.) Quél. and *Phellinus weirii* (Murr.) Gilb. (Nobles, 1948, 1965; Childs, 1963; Boidin, 1971; Hansen, 1979a, 1979b). Mechanisms for genetic diversity in *P. noxius* have not been examined previously. A study of nuclear mechanisms operating in *P. noxius* would be useful not only for understanding variation in the species, but also as a contribution towards knowledge of such mechanisms in the Hymenochaetaceae.

The studies reported herein investigated some aspects of variation in *P. noxius*. The project comprised two parts:

(i) Chapters 3-5. Field isolates from two hosts and a range of geographical localities were examined for variation in cultural features, temperature-growth and pH-growth relations, production of extracellular enzymes and pathogenicity; the limits of variation among monosporous isolates derived from a single field isolate were established to give a better appreciation of the overall variation found among the field isolates.

(ii) Chapters 6-7. Explanations for the variability encountered in (i) were sought by investigating the nuclear life history and sexual and vegetative incompatibility in the fungus.

CHAPTER 2

MATERIALS AND METHODS

2.1 SOURCES AND PREPARATION OF ISOLATES

Isolates of *P. noxius* were derived from field collections (referred to as "field isolates" in the text), and from single basidiospores and arthrospores.

Field isolates. These isolates came from infected tissues of hosts ("host isolates"), contexts of basidiocarps ("context isolates") and sterile encrustations of *P. noxius* found on the roots and butts of infected trees ("encrustation isolates"). The origins of all field isolates used in the present studies are given in Table 2.1: two hosts (hoop pine and rubber) and four geographical regions (Sri Lanka, Malaysia, north Queensland and southeastern Queensland) are represented. Queensland isolates were originally grown on malt-extract agar (MEA) containing 2 per cent (w/v) malt-extract and 2 per cent agar, but were later maintained on MEA containing 1.25 per cent malt-extract. Generally, antibiotics were not required to suppress contaminating fungi during isolations, but where used, benomyl (1-butyl carbamyl-2-benzimidizole carbamic acid methyl ester) was incorporated into the medium at 5 µg/l (Taylor, 1971; Maloy, 1974).

Table 2.1 Origins of field isolates

Isolate number	Origin	Month/Year isolated
517A/1	Decay, hoop pine stem, S.E. Queensland	10/68
576B/1	Decay, hoop pine stem, S.E. Queensland	2/69
604/2	Basidiocarp on hoop pine, N. Queensland	5/69
1075/2	Root of hoop pine, S.E. Queensland	9/72
1141C	Root of hoop pine, S.E. Queensland	3/73
1166A	Encrustation on hoop pine, S.E. Queensland	6/73
1166B/4	Decay, hoop pine stem, S.E. Queensland	6/73
1167	Encrustation on hoop pine, S.E. Queensland	6/73
1354B	Root of hoop pine, S.E. Queensland	5/74
1516	Basidiocarp on hoop pine, S.E. Queensland	6/74
1518	Basidiocarp on hoop pine, S.E. Queensland	6/74
2002	Basidiocarp on hoop pine, S.E. Queensland	5/75
2250	Basidiocarp on hoop pine, N. Queensland	7/75
2261	Basidiocarp on hoop pine, N. Queensland	7/75
LB1	Root of rubber, Sri Lanka	12/76
LB2	Basidiocarp on rubber, Malaysia	4/56
LB3	Root of rubber, Malaysia	4/77
LB4	Encrustation on rubber, Malaysia	9/77
LB5S	Basidiocarp on hoop pine, N. Queensland	3/78
LB5W	Decay behind basidiocarp LB5S, N. Queensland	3/78

Isolates 517A/1 to 2261 inclusive are from the culture collection of the Queensland Department of Forestry; isolate LB1 is culture RRISL410 from the Rubber Research Institute of Sri Lanka; and isolates LB2, LB3 and LB4 are, respectively, cultures RRIM531, 1228 and 1229 from the Rubber Research Institute of Malaysia.

Monobasidiospore Isolates. All attempts to harvest spores from sporulating basidiocarps detached from their substrates were unsuccessful but the following procedure provided a reliable source of basidiospores. A 60 cm long trunk section bearing applanate basidiocarps of *P. noxius* with fresh hymenia was removed from a 35 year old hoop pine in a north Queensland plantation. A hole, 2.5 cm diameter and 10 cm deep, was drilled

halfway along each of three radii at the top end of the section. The section standing on its butt end on sterilized sandy loam in an aluminium tray was placed in an Email LBH controlled environment cabinet. The loam was saturated and the holes were filled with distilled water. Continuous light was provided by fluorescent tubes emitting a 4 00 - 7 00 nm wavelength spectrum at an intensity of 350-400 $\mu E\ m^{-2}sec^{-1}$. Conditions of moderate temperature, low light and high humidity prevailing in the plantation from which the stem section was collected were simulated by setting temperature in the cabinet at $25^{o}C$, and by covering the section and loam with a black polythene envelope into which a 15 cm x 5 cm clear polythene window was sewn in a position above the basidiocarps. Water in the holes and loam were replenished daily for the first week, and every two or three days thereafter. Under these conditions, basidiocarps sporulated continuously for five months.

Basidiospores were collected overnight in a small glass petri dish of distilled water suspended below a basidiocarp. Larger debris was removed by passing the spore suspension through an 8 µm Millipore filter (SCWP 025 00) in a Swinny adapter (Millipore Company) fitted to a syringe. Spores were recovered by passing the filtrate through an 0.25 µm filter (HAWP 025 00). To reduce bacterial contamination, spores were washed three times by passing 20 ml sterile distilled water through the filter. The filter disc carrying the spores was transferred to a sterile 15 cm x 2.5 cm test tube, and 10 ml sterile water were added. After vigorous shaking, the concentration of spores in suspension was determined with a Spenser Bright-Line Hemacytometer with improved Neubauer rulings (American Optical Company). The spore concentration was adjusted to 1,300-1,600 spores/ml with sterile distilled water.

Two streaks of diluted spore suspension were applied with a nichrome

wire loop to the surface of MEA (1.25% malt-extract) in 90 mm plastic petri dishes containing 10 ml medium. Dishes were incubated at 25°C in the dark for 48 hr, and then examined from below under 100X magnification. The locations of well-separated monobasidiospore colonies were marked on the underside of each dish. Generally, four or five such colonies were found on each streak. Discs 4 mm diameter, each with a single colony, were cut from the medium and transferred to separate dishes of fresh MEA. Seven days later, isolates were placed in a culture collection.

Monoarthrospore Isolates. A context isolate (LB5S) was obtained from the basidiocarp which produced the spores for single-basidiospore isolations. To prepare monoarthrospore isolates, a 15 cm x 2.5 cm test tube containing 15 ml MEA (1.25% malt-extract) was inoculated with LB5S, and placed in the dark in an incubator at 25°C. After four days, 10 ml of sterile distilled water were added to the tube, and to avoid fungal material other than arthrospores being taken into suspension, was shaken only gently. The suspension was then transferred to another sterile tube and shaken vigorously until microscopical examinations revealed that spores were no longer attached in chains. The procedure for preparation of single-arthrospore isolates from such suspensions was similar to that used to prepare monobasidiospore isolates, except that streaked dishes were incubated for only 24 hr. Monoarthrospore isolates were freshly prepared for each of the major studies.

2.2 DESCRIPTION OF CULTURES

Like many other plant-pathogenic and wood-decaying Hymenomycetes (Nobles, 1965), *P. noxius* does not form basidiocarps in routine agar culture (Bakshi *et al.*, 1970; Bolland, pers. obs.), and must be identified

from its cultural characters. The procedure originally proposed by Nobles (1948), or a later modification (Nobles, 1965), are used most often to describe or identify isolates of such fungi (Nobles, 1965; Bakshi *et al.*, 1969, 1970; Niemelä, 1970, 1971, 1972, 1974, 1975, 1977b; Setliff, 1972; Sen, 1973; Niemelä & Ryvarden, 1975). Nobles' modification was adopted to describe cultures in the present studies. The macroscopic and microscopic features of isolates grown on malt-extract agar, and the effect of the fungus on the malt-extract agar and on malt-extract agar containing gallic or tannic acid, were characterized in accordance with the definitions and illustrations of Nobles (1965).

The "Key Code" for *P. noxius* was determined also. Nobles (1965) ascribed numbers (Code Symbols) to characters which she considered to have diagnostic value; the series of Code Symbols expressing the cultural characters of an isolate constitutes the Key Code for a species.

A multiple-choice key of Key Codes compiled from studies on isolates from authenticated basidiocarps is used when an isolate is to be identified.

2.3 MEASUREMENT OF GROWTH AND LEVEL OF REPLICATION

Growth of *P. noxius* on malt-extract agar under various conditions of temperature and pH, was assessed in 90 mm diameter petri dishes. This method was selected following experiments testing three methods for assessing growth: (i) mycelial dry weight production in liquid culture, (ii) linear growth rate in growth tubes, and (iii) radial growth rate in petri dishes (Appendix 4). Although variation among replicates was least in the mycelial dry weight method (Appendix 4), observations on growth in petri dishes appeared more suitable for the study of variation within and between isolates. Sectors of different cultural types arose in petri dish culture, and these types differed in growth rate; they

might differ also in other physiological traits.

Two phases of growth were recognized in the early development of cultures of *P. noxius* in petri dishes: (i) a phase of accelerating growth, followed by (ii) a phase of linear growth. The latter commenced on the third day of observable growth, and time of initiation was independent of both the isolate studied and the temperature of incubation. In the present project, radial growth rates were assessed during the linear phase of growth.

Studies on the level of replication required (Appendix 4) revealed that the accuracy of the data was not improved when the number of replicates in a treatment was increased from four to eight. Mean radial growth rates of cultures in individual treatments were determined from three radii in each of four petri dishes.

2.4 INOCULA FOR STUDIES ON pH-GROWTH RELATIONSHIPS

Plugs of malt-extract agar, carrying mycelia of *P. noxius*, were used as inocula in studies on temperature-growth relations. The use of such inocula in pH-growth studies, would probably permit growth at pH values which would normally be inhibitory to the fungus, i.e., the inoculum substrate would support some initial growth. Inocula for these studies therefore comprised flecks of aerial mycelia taken from cultures grown on malt-extract agar containing 15 per cent malt-extract. The medium promoted profuse development of aerial mycelia, and thus mycelial flecks free of supporting substrate could be obtained, if removed carefully.

Preliminary studies (Appendix 5) showed that growth rates in dishes inoculated with mycelial flecks could be assessed using the same procedure as that employed in experiments using agar plugs.

2.5 ASSAYS FOR EXTRACELLULAR ENZYMES

These studies concentrated on an initial screening of a large number of isolates of *P. noxius* for variation in the production of a range of extracellular enzymes. Qualitative assays on diagnostic media were considered more appropriate than sophisticated quantitative determinations. Also, they afford an opportunity to consider the merits of proposals by Taylor (1974, 1977) and Hankin and Anagnostakis (1975) that such assays be used to differentiate fungal taxa.

The test substrates of Hankin and Anagnostakis (1975, 1977) were adopted for the studies on *P. noxius* because they were used also by Raper and Kaye (1978) in investigating the variation in enzyme production among *Agaricus* spp. The results for *P. noxius* can therefore be compared with those for other species in two separate studies where the same substrates were used. The substrates of Taylor (1974, 1977) apparently have not been used by other workers to date.

2.6 PATHOGENICITY STUDIES

2.6.1 General Method

The pathogenicity of hymenomycete root pathogens has been studied (i) in the field (where either seedlings have been planted in infection centres, or roots of already established plants have been directly inoculated), (ii) in the glasshouse, or (iii) in the laboratory (where aseptic seedlings have been inoculated on synthetic media). Generally, field and glasshouse inoculations have been unsuccessful in demonstrating differences in the pathogenicity of isolates, or in the susceptibility of hosts, because percentages of plants infected have been low, or

because results for individual isolates or hosts have varied widely (Whitney, 1963, 1972; Raabe, 1967; Kuhlman, 1969, 1970; Dimitri, 1974; Greig, 1974). In contrast, laboratory inoculations have been generally successful; per cent infections are usually high, and significant differences between isolates, or hosts, are usually recorded (Hüppel, 1970; Koenigs, 1970; Whitney & Bohaychuk, 1976, 1977). These generalizations are supported by the studies of Lane and Witcher (1974) in which *Pinus echinata* Mill. was inoculated with *Heterobasidion annosum* in the field, glasshouse, and laboratory. In this instance, per cent infections in laboratory inoculations were high and significant differences were recorded between isolates, infections were not recorded in the field, and low and variable infections were recorded in the glasshouse. Thus, in the present studies on *P. noxius,* seedlings of test plants were inoculated in aseptic laboratory culture.

2.6.2 Selection of Test Species

Phellinus noxius has been recorded as a root pathogen on a wide range of species from both divisions of the Spermatophyta, and hosts include herbaceous plants, shrubs and trees (Chapter 1). Hence, for the present studies, a selection of three test species was made from diverse groups of plants, viz., an herbaceous plant, a conifer and a hardwood.

New Zealand blue lupin (*Lupinus angustifolius* L.) was selected as the herbaceous plant as seeds were available in the laboratory.

Hoop pine (*Araucaria cunninghamii*) was considered as the representative of conifers because of the importance of the fungus in plantations of this species in Queensland (Chapter 1). However, repeated attempts to raise uncontaminated seedlings were unsuccessful. Seeds from three separate lots bore pustules of an unidentified Hyphomycete, and although

individual seeds were screened for obvious infection, and severe surface-sterilization techniques were used, contamination always occurred. Hence, Monterey pine (*Pinus radiata* D.Don) was selected as an alternative.

Queensland grey ironbark (*Eucalyptus drepanophylla* F.Muell. ex Benth.) was selected as the hardwood because it is one of the two species of this important Australian hardwood genus which have been recorded as hosts of the fungus (Appendix 2).

2.6.3 Assessment of Pathogenicity

The pathogenicity of isolates of hymenomycete root pathogens on their hosts has been determined quantitatively in various ways, e.g. (i) the percentage of inoculated roots or plants infected (Whitney, 1963, 1964; Raabe, 1967, 1972), (ii) per cent mortality (Kuhlman, 1969, 1970; Koenigs, 1970; Lane & Witcher, 1974; Lauska, 1974) and (iii) virulence (or disease) ratings (or indices), which took into account the rapidity with which an isolate induced either symptoms or mortality (or both) in individual seedlings (Raabe, 1967, 1972; Kuhlman, 1969, 1970; Whitney & Bohaychuk, 1976, 1977; James & Cobb, 1978).

Two assessments of pathogenicity were used in the present studies: (i) per cent mortality, and (ii) virulence. The former recorded the number of seedling deaths at the conclusion of the study, and the latter, the rapidity with which an isolate induced initial symptoms of disease, and then mortality, in seedlings. None of the indices or ratings used in the studies cited above seemed appropriate for the present studies. A number of formulae for virulence were therefore devised and tested, and the following was considered the most satisfactory:

$$V.I. = S_n + M_n,$$

where V.I. is the virulence index, and S_n and M_n are numerical values ascribed to the period from inoculation to production of the first symptom of disease, and mortality, respectively, for each seedling. These periods, and the relevant numerical values ascribed to them, are given in Tables 2.2 and 2.3, respectively.

Table 2.2 Time scales (days after inoculation) used in assessing the virulence index of an isolate on a seedling

Period	Lupin	Pine and Ironbark
I	1-7 days	1-14 days
II	8-14 days	15-28 days
III	15-21 days	29-42 days

Table 2.3 Numerical values ascribed to time scales (see Table 2.2) in which initial disease symptoms or death was induced in a seedling

Period	First Symptoms (S_n)	Mortality (M_n)
I	3	6
II	2	4
III	1	2

Example: If a lupin seedling displayed first disease symptoms nine days after inoculation, and was recorded as dead seven days later, the virulence index for the isolate on the seedling would be:

$$= S_n + M_n$$
$$= 2 + 2 = 4 \text{ (from Tables 2.2 and 2.3).}$$

The maximum index for any seedling would be: $= 3 + 6 = 9$.

2.7 NUCLEAR STUDIES

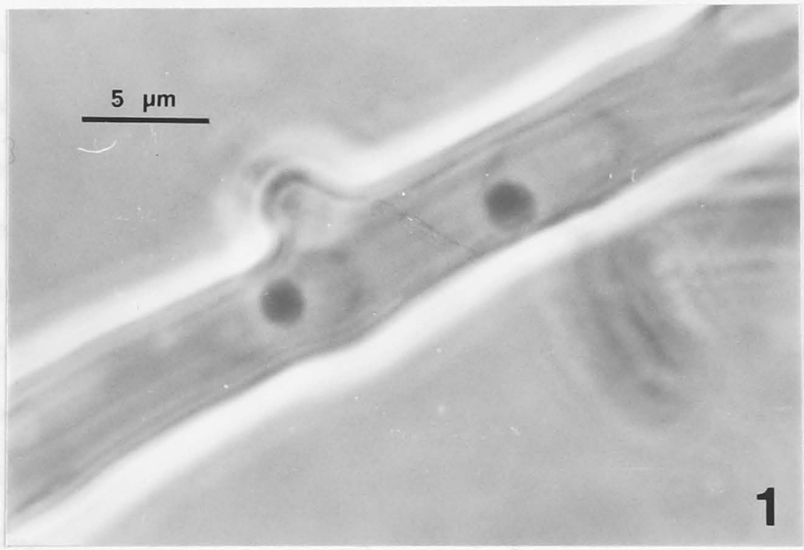
2.7.1 Morphology and Behaviour

Fungal nuclei usually cannot be observed with bright field microscopy. (i) Phase contrast microscopy, (ii) nuclear staining prior to observations with fluorescence or bright field microscopy, and (iii) transmission electron microscopy are the usual techniques employed to study fungal nuclei. The latter technique is used primarily to study the ultrastructure of nuclei and nuclear division (Setliff *et al.*, 1974; Powell, 1975; Forer, 1978; Girbardt, 1978; Heath, 1978); such studies, however, are outside the scope of this project.

Phase contrast microscopy has been used successfully in observations on gross nuclear morphology and behaviour (migration, fusion and division) in living cells of Hymenomycetes such as *Armillariella mellea* (Korhonen & Hintikka, 1974), *Clitocybe trunicola* Peck (Bistis, 1970), *Heterobasidion annosum* (Aist & Wilson, 1968), and *Schizophyllum commune* Fr. (Parag, 1968). However, the technique was unsatisfactory for *P. noxius* as the granular nature of the protoplasm precluded ready recognition of nuclei in hyphae of the fungus (Figure 2.1). Nomarski interference contrast microscopy was similarly unsuccessful.

In the present studies, observations on nuclei were confined to stained material. The stains most widely used for fungal nuclei appear to be Feulgen, Giemsa and haematoxylin. Frequently, a combination of stains has been used to study the nuclei of a fungus (e.g. Hrushovetz, 1956; Robinow, 1957; Bakerspigel, 1959; Knox-Davies, 1967; Tommerup & Broadbent, 1975) because various stains differ in their specificities for the components of the nucleus. Feulgen and Giemsa stains apparently

Figure 2.1 Hyphae of *Schizophyllum commune* (1) and *Phellinus noxius* (2) viewed with phase contrast microscopy. The nuclei of *S. commune* are clearly visible and show prominent nucleoli, whereas those of *P.noxius* are not obvious in the granular cytoplasm.


5 µm
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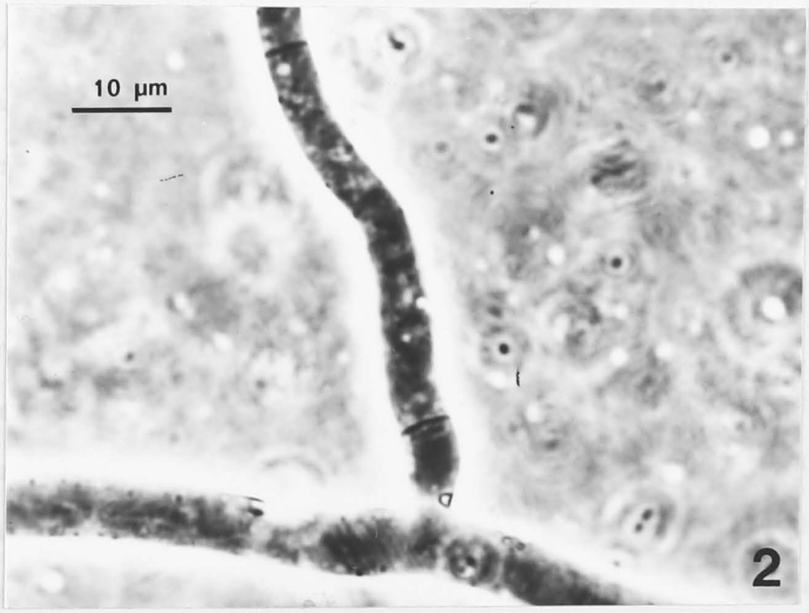
10 µm
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stain chromosomes well (Hrushovetz, 1956; Knox-Davies, 1967; Bryant & Howard, 1969; Brody & Williams, 1974; Galeotti & Williams, 1978); however, nucleoli do not take up the stain (Robinow, 1957). Haematoxylin stains nucleoli and centrioles, but not chromosomes (Robinow, 1957; Bakerspigel, 1959). Bakerspigel (1959) considered that haematoxylin is not useful for studying dividing nuclei in fungal cells. All three stains were used in the present studies.

A procedure developed for staining nuclei of one fungus may not be applicable for other fungi. For example, numerous modifications of the technique for Giemsa staining have been described by various authors (Hrushovetz, 1956; Knox-Davies & Dickson, 1960; Ward & Ciurysek, 1961; Duncan, 1970; Brody & Williams, 1974; Korhonen & Hintikka, 1974; Setliff *et al.*, 1974; Tommerup & Broadbent, 1975; Delgardo & Cook, 1976). All these modifications were found to be unsatisfactory for use with *P.noxius*. The staining process comprises a complex sequence of treatments: fixation, hydrolysis, staining, clearing cytoplasm of excess stain, dehydration and mounting. A satisfactory procedure was developed for Giemsa staining nuclei in vegetative mycelia of *P. noxius*, but only after extensive experimentation with different chemicals for fixation and hydrolysis, and with different periods for fixation, hydrolysis and staining. A similar series of experiments was undertaken to develop a procedure for staining with Feulgen.

A number of Schiff's reagents are available for the technique, for example, acriflavine, acridine yellow, coriphosphine, auramin-O, and pararosaniline hydrochloride (Böhm & Sprenger, 1968; Bryant & Howard, 1969; Lemke *et al.*, 1975; Peabody *et al.*,1978); but only the latter (as basic fuchsin) was tested. Considerable problems were encountered with the technique. Staining of satisfactory intensity was achieved in only

one staining session; however, this was not reproducible despite the use of an identical procedure and solution of Schiff's reagent. The difficulties associated with the use of Feulgen stains have been recognized elsewhere (Forche & Blaschke, 1978). The method of Chang (1977) was found to be satisfactory for staining nuclei of *P. noxius* with haematoxylin. Details of the staining procedures used are given in the sections (6.2.2, 6.3.2 and 6.4.2) of "Materials and Methods" in Chapter 6.

2.7.2 Determinations of Ploidy of Nuclei

The occurrence of nuclei of different ploidy in the vegetative phase of the one fungus has been demonstrated elsewhere, usually by one or more of the following methods: (i) counts of chromosomes at prophase to metaphase of mitotic division (e.g. Knox-Davies & Dickson, 1960; Hosford & Gries, 1966; Knox-Davies, 1967; Brody & Williams, 1974; Wright & Lennard, 1978), (ii) direct measurements of the DNA content of nuclei in spores (e.g. Ishitani *et al.*, 1956; Olson & Borkhardt, 1978), (iii) determinations of spore volumes, where diploid spores are approximately double those of haploid spores (e.g. Clutterbuck, 1969; Burnett, 1975), (iv) measurements of nuclear diameter, or determinations of nuclear volume (e.g. Hosford & Gries, 1966; Little & Manners, 1969; Tommerup & Broadbent, 1975; Williams, 1975; Williams & Mendgen, 1975; Olson & Borkhardt, 1978), and (v) microspectrophotometric determinations of the relative DNA content of nuclei (e.g. Böhm & Sprenger, 1968; Bryant & Howard, 1969; Ahrberg, 1975; Williams & Mendgen, 1975; Haskins, 1977; Peabody *et al.*, 1978). Attempts were made to use all except (ii) of these methods, in the present investigations. Methods (iii) and (v)

require further explanation.

Preliminary observations on nuclei in vegetative mycelia and asexual spores, made during the experiments on developing satisfactory procedures for staining (Section 2.7.1), revealed that both the number and size of nuclei varied widely within spores of the one culture of *P. noxius*. This variation in nuclear size possibly reflected variation in the ploidy of the nuclei. If nuclei in the spores of *P. noxius* were all of the same ploidy, it would be expected that spore volume would be closely correlated with the number of nuclei. However, if they differed in ploidy, then spore volume would probably be poorly correlated with the number of nuclei, but closely correlated with nuclear volume per spore. These correlations were examined in the present studies.

Microspectrophotometric determinations of DNA content are made on nuclei stained with Feulgen (Schiff's) reagents. These reagents are not only specific for DNA, but they also react stoichiometrically with it. Hence, the quantity of stain retained by a nucleus (i.e. the intensity of staining), after the cytoplasm has been cleared of excess stain, is directly proportional to the DNA content of the nucleus. The intensity of the staining of a nucleus is measured with a photometer fitted to the microscope, either by fluorescing the stain (with blue, green, or UV excitation filters), or by absorption of a predetermined wavelength (i.e. one which gives maximum absorption values for the stain used). A facility was available for the latter method.

2.8 STUDIES ON SEXUAL AND VEGETATIVE INCOMPATIBILITY: BACKGROUND AND TERMINOLOGY

The following, except where otherwise indicated, is taken from Raper (1966) or Burnett (1975, 1976).

2.8.1 Sexual Incompatibility

The Life Cycle of Holobasidiomycetes. The typical life cycle consists of the following sequence of stages and events: (i) a uninucleate basidiospore, borne on a basidiocarp, germinates to produce (ii) a monokaryotic mycelium containing uninucleate cells of genetically identical haploid nuclei. Two compatible monokaryons fuse giving rise to (iii) a dikaryotic mycelium containing cells with two genetically dissimilar nuclei originating from the two monokaryons. The dikaryotic mycelium is capable of indefinite vegetative propagation, the dikaryotic condition being maintained by synchronous division of the two nuclei and the formation of a hook cell. The hook cell soon fuses with the subterminal cell; the clamp connection, the remains of the fused hook cell, characterizes the dikaryon. Under appropriate environmental conditions, the dikaryon bears (iv) a basidiocarp comprising dikaryotic mycelia. (v) A specialized fertile tissue (the hymenium) forms upon or within the basidiocarp and consists of terminal, spore-bearing cells, (vi) the basidia. In each basidium, the dikaryotic pair of nuclei fuse to form (vii) a transient diploid nucleus. (viii) Meiosis follows immediately to produce four haploid nuclei which migrate into (ix) four basidiospores formed exogenously on the basidium.

Whereas the above applies to the majority of known species, variations in several of the stages and events occur in other species (Raper, 1978).

Mating Systems. Sexually breeding fungi are usually homothallic or heterothallic. Homothallic fungi are self-fertile; they complete the sexual cycle on a single homokaryotic (i.e. nuclei are genetically identical, but cells may contain more than one nucleus) mycelium. About

10 per cent of investigated holobasidiomycetes are homothallic. The remaining species require the interaction of two genetically dissimilar, but compatible, homokaryons to complete the sexual cycle. Two distinct incompatibility systems operate among such fungi, namely, bipolar (or unifactorial) and tetrapolar (or bifactorial) incompatibility.

Bipolar incompatibility is characterized by a single incompatibility factor, the A factor; and the dikaryon is only formed when fusion occurs between two homokaryons carrying different A factors. Following nuclear fusion in the basidium, the two factors segregate in meiosis giving rise eventually to basidiospores in the one tetrad, and in the one basidiocarp, with parental factors in equal frequency. The essential points of bipolar incompatibility may be represented thus:

Homokaryons		Dikaryons		Diploid		Homokaryotic progeny
A1 x A2	⟶	(A1 + A2)	⟶	A1A2	⟶	A1; A1; A2; A2
	mating		fusion		meiosis	

Hence, interbreeding among siblings is restricted to 50 per cent. It is estimated that 25 per cent of holobasidiomycetes have bipolar incompatibility.

The remaining holobasidiomycetes (i.e. about 65% of species) have tetrapolar incompatibility. Incompatibility is determined by two factors, A and B, which recombine and segregate in meiosis; and therefore, basidiospores of four mating types are produced in equal frequency:

Homokaryons		Dikaryons		Diploid		Homokaryotic progeny
A1B1 x A2B2	⟶	(A1B1 + A2B2)	⟶	A1B1A2B2	⟶	A1B1; A2B2; A1B2; A2B1
	mating		fusion		meiosis	

The dikaryon is established only when two homokaryons carry different

A and B factors. Interbreeding among siblings is restricted to 25 per cent. Hence, inbreeding is more restricted in tetrapolar than in bipolar incompatibility.

Morphology of Interactions. Laboratory determinations of mating systems rely upon the recognition of compatible and noncompatible interactions between paired monobasidiospore mycelia. Most reports on sexual incompatibility in the Holobasidiomycetidae concern species which have septal clamp connections on dikaryotic hyphae. Hence, the following descriptions of interactions recorded in various mating systems in the sub-class are based almost entirely on such species.

Homothallic species are either primarily homothallic (i.e. the sexual cycle is completed on mycelia derived from basidiospores containing a single haploid nucleus) or secondarily homothallic (basidiospores contain two haploid nuclei with compatible mating factors). Nearly all investigated homothallic species are secondarily homothallic. Primary homothallism has apparently been demonstrated conclusively in *Coprinus sterquilinus* Fr. Uninucleate basidiospores give rise to mycelia constituted of binucleate cells with septal clamp connections; and if a uninucleate subterminal cell is isolated, the dikaryotic condition is re-established within a very few nuclear divisions. Most secondarily homothallic species investigated produce only two binucleate spores per basidium, although some 4-spored (each binucleate) species are known (Ginns, 1974).

Compatible interactions between homokaryons can be recognized in bipolar and tetrapolar species by any or all of the following characters: septal clamp connections are formed; the cultural appearance of dikaryotized mycelia differs from that of the homokaryons and resembles the dikaryon or "wild type"; and normal, fertile basidiocarps are produced.

Clamp connections are not formed when noncompatible or hemicompatible homokaryons are paired.

In noncompatible pairings within bipolar species, the cultural appearance of the homokaryons is not altered, and morphologically distinct interactions may occur along the zone of confrontation between cultures. For example, common A (A=) matings in *Fomes cajanderi* Karst. are recognized by a distinct aversion zone (or "barrage" - Raper, 1966) in which there is a dark pigmentation in the agar, or much less frequently, by a free intermingling of hyphae and no discoloration of the agar (Neuhauser & Gilbertson, 1971).

Those in *Heterobasidion annosum* are recognized by a distinct "barrage" of sparse mycelium (Korhonen, 1978b). However, in *Polyporus palustris* Berk. & Curt. the "barrage" comprises mounded mycelia, or the "barrage" may be absent and hyphae simply overlap (Raper, 1966).

Within tetrapolar species, up to three distinct interactions have been recorded between hemicompatible or noncompatible homokaryons. For example, in *Schizophyllum commune* a "barrage" of sparse mycelium occurs along the zone of confrontation where the B factors in both homokaryons are identical (i.e. A≠B=); both homokaryons are converted to heterokaryons with sparse, appressed mycelia where the A factors are identical (A=B≠); and there is a narrow zone of mutual overgrowth of the two mycelia, and the cultural appearance of the homokaryons remains unchanged where both factors are identical (A=B=) (Raper, 1966). The morphology of these interactions may differ in other species of clamped holobasidiomycetes (Raper, 1966). The interpretation of interactions may be difficult as evidenced by the reclassification by Ginns (1974) of several species originally designated as either bipolar or secondarily homothallic bipolar. If such errors can be made with species

which have septal clamp connections on dikaryotic mycelia, the interpretation of interactions in species which lack clamp connections may be even more difficult.

Interfertility. While compatible interactions among siblings from the one basidiocarp may be restricted to 50 per cent of pairings in bipolar species, and 25 per cent in tetrapolar species, any sibling from the one basidiocarp may be compatible with all those from another basidiocarp. This is due to the multiallelic nature of the mating factors. Such strains are thus completely interfertile. However, a number of hymenomycete species (and others) have evolved strains on different hosts or in different localities which are completely intersterile. Among hymenomycete root pathogens, for example, Kornonen (1978b) has identified at least three intersterile groups among isolates of *H. annosum* from world-wide sources. Two of the groups occur in Finland (viz. the "pine" group and the "spruce" group), and a third occurs in Australia, New Zealand and Fiji. Raper (1966) and Burnett (1976) give examples of other species of Hymenomycetes in which intersterility among strains has been demonstrated.

Interfertility among isolates may be tested by two methods. In one, monobasidiospore mycelia are paired. In the other, interfertility can be determined with the aid of the Buller phenomenon (Raper, 1966): homokaryotic mycelia are dikaryotized by dikaryotic mycelia of another compatible strain; and so, monobasidiospore mycelia may be paired with synthesized dikaryons, or with isolates from basidiocarps or host tissues. As mentioned previously (Section 2.1), monobasidiospore isolates used in the present project came from only the one basidiocarp. Hence, the latter method was used in tests for interfertility reported herein.

2.8.2 Vegetative Incompatibility

In sexual incompatibility, mating is prevented between mycelia carrying the same factor(s); but in vegetative incompatibility, it is restricted between strains which have different factor(s). Vegetative incompatibility allows adapted genotypes to emerge and predominate in a particular ecological niche, and prevents the exploitation of these genotypes by those which are less well adapted. Hyphal fusions may never be wholly suppressed, even between different fungal species, but their frequency can be greatly reduced by morphological, physiological, or other types of "sterility barriers". In the Hymenomycetes, these "sterility barriers" may be manifested in laboratory culture by a variety of morphological interactions. For example, mycelia derived from genetically different strains may form mounds of mycelia, or darkly pigment the agar (or both) along the zone of confrontation (Brodie, 1936; Childs, 1963; Adams & Roth, 1967; Barrett & Uscuplic, 1971; Kemp, 1974; Burnett, 1976; Shaw & Roth, 1976; Hansen, 1979b). Hansen (1979b) reviewed the terminology describing these interactions, and preferred "line of demarcation" as it can be used unambiguously in this context. The term is therefore adopted for the present studies to describe interactions recorded for vegetative incompatibility in *P. noxius*.

2.9 SCANNING ELECTRON MICROSCOPY

Specimens were placed on glass coverslips and frozen with liquid nitrogen, and then transferred to the pre-cooled ($-60^{o}C$) stage of a Dynavac Tissue Freeze Drier and subjected to approximately 10^{-1} Torr vacuum for 24 hr. They were then fixed to aluminium stubs with nail

varnish and coated with approximately 3,000 nm gold in a Dynavac vacuum coating unit. A Cambridge Stereoscan II scanning electron microscope operating at 30 kV accelerating voltage, was used to examine the specimens. Photographs were taken with Kodak Plus X film.

2.10 STATISTICAL METHODS

Comparisons of means were made using the methods recommended by Bishop (1966), and were based on the assumption that samples were drawn from populations with different standard deviations. The significance of differences between means were assessed at $p = 0.05$. Where undertaken, analyses of variance were made with the ANOVA sub-programme of the statistical package for the social sciences (Nie *et al.*, 1975).

2.11 QUALITY OF CHEMICALS

All chemicals used were of analytical grade, with the exception of gallic and tannic acids, gum guaiac, and the nuclear stains.

CHAPTER 3

VARIATION IN CULTURAL CHARACTERS

3.1 INTRODUCTION

The only published description of the cultural characters of *P. noxius* was based on a single isolate from decayed wood (Bakshi *et al.*, 1970). Further studies on the cultural characters of isolates from a variety of sources, including basidiocarps, would assist identification, and permit studies on the relationship of variability in other aspects of the biology of the fungus to cultural type.

The following experiments describe the cultural characters of *P. noxius*, and investigate the variation in these characters within and among isolates of the fungus. Isolates studied came from host and fungal tissues, and from single asexual and sexual spores.

3.2 EXPERIMENT 1: DESCRIPTION OF CULTURAL CHARACTERS

3.2.1 Materials and Methods

Twenty field isolates, 23 monobasidiospore isolates, and 20 monoarthrospore isolates were studied. The origins of the isolates, and methods for their preparation are given in Section 2.1. Field and monobasidiospore isolates were taken from a culture collection maintained on malt-extract agar (MEA: 12.5 g Difco Bacto malt-extract; 20.0 g Difco Bacto-Agar; 1000 ml distilled water), but monoarthrospore isolates were freshly prepared.

A modification of the procedure developed by Nobles (1965) for recording the cultural characters of wood-destroying Hymenomycetes was adopted for the experiment. Inoculum cultures were prepared by growing field and monobasidiospore isolates for 4 days, and monoarthrospore isolates for 7 days, from central inoculations in petri dishes of MEA. One 4 mm diameter plug, cut from the advancing margin of an inoculum culture, was placed mycelium down at the edge of a 90 mm diameter petri dish containing 30 ml MEA. Six dishes per isolate were inoculated and then incubated for 6 weeks at 25°C in the dark.

At weekly intervals, cultures were removed from the incubator and the following macroscopic characters were recorded: growth rate (expressed as the number of weeks required by the fungus to overgrow the surface of the medium); form and character of the advancing zone; colour, topography, and texture of the mat; presence of basidiocarps; colour changes in the medium induced by the fungus, as observed from the underside of the dish; and odour. Descriptions of all characters except mat colour were based on the terminology of Nobles (1948, 1965). Mat colours were described according to the mycological colour chart of Rayner (1970), and appear as capitalised names in the text. Cultures of selected isolates were photographed at 1 and 6 weeks with an Asahi Pentax KM camera using Kodak Panatomic X film.

At 1, 3 and 6 weeks, aerial and submerged mycelia from the advancing margin and older mat were mounted in lactophenol-cotton blue on a glass slide to record, describe and measure the microscopic characters. Because over 300 cultures were studied, it was necessary to prepare permanent mounts for examination as time permitted. These mounts were prepared using the technique of Omar *et al.* (1978): a drop of polyvinyl alcohol was added to the lactophenol-cotton blue just before placing the coverslip.

Mycelium was also mounted in 5 per cent aqueous KOH to test for colour change. Slides were examined with a Wild M20 microscope employing bright field optics, and the illustrations of Nobles (1965) were used to identify the characters observed. Measurements were made using an eyepiece scale calibrated with a stage micrometer. Selected material was photographed with a Carl Zeiss photomicroscope using bright field optics and Kodak Panatomic X film.

Extracellular oxidase production by the fungus was tested by the Bavendamm method as modified by Davidson *et al.* (1938) and by the procedure developed by Nobles (1958). In the former test, a culture was grown from a central inoculation in a 90 mm diameter petri dish containing 30 ml gallic acid or tannic acid agar (GAA and TAA, respectively: 15.0 g Difco Bacto malt-extract; 20.0 g Difco Bacto-Agar; 1000 ml distilled water; 5.0 g gallic (or tannic) acid). Two dishes of each medium were inoculated with each isolate, and then incubated for 7 days at 25^{o}C in the dark. The intensity of diffusion zones indicating the production of extracellular oxidase, and measurements of growth along four radii were recorded for each culture. In the latter test, drops of an alcoholic solution of guaiacum (0.5 g gum guaiac in 30 ml 95% ethanol) were placed on the mats of 3 week old cultures grown on MEA, at three positions between the margin and inoculum. The rapid appearance (2-4 min) of a blue colour indicated the presence of the oxidase; lack of change, or a slow appearance of a pale blue colour, indicated its absence.

A Key Code was prepared for *P. noxius* using the Code Symbols devised by Nobles (1965). The role of the Key Code in the identification of Hymenomycetes was discussed in Section 2.2.

Observations on field isolates were repeated in three separate

studies over a period of 12 months, but those on monosporous isolates were made in only a single study.

3.2.2 Results

3.2.2.1 Field Isolates

In routine maintenance subculturing and in the preparation of inoculum cultures for these studies, two cultural types were recognized in nine of the 20 isolates. They were temporarily termed the Pigmented and Unpigmented type. Cultures of the former type were characterized macroscopically by the occurrences of pigmented mycelial mats and a distinct brown coloration in the agar. Mats of cultures of the latter type developed no noticeable pigmentation, and the agar retained its normal coloration.

Cultural Characters of the Pigmented Type

Macroscopic Characters

The fungus grew rapidly and covered the surface of the medium in 8-14 days. The advancing margin was white, uneven, raised, cottony and 10-15 mm wide (Fig. 3.1).

The older mat was initially white and cottony to woolly but soon collapsed becoming sub-felty to farinaceous felty. Pigmentation of the mat usually developed within 7 days. Initially it was Pale Luteous and then it progressed through Luteous to Sienna. Occasionally, faster growing unpigmented sectors arose from advancing pigmented mats, but they developed pigmentation soon after the substrate was overgrown. The area of mat pigmentation in 6 week old cultures varied considerably (Fig. 3.2); cultures were mostly unpigmented (e.g. Figs. 3.2.12 and 3.2.10), mostly pigmented (Figs. 3.2.6, 3.2.8), or entirely pigmented (Figs. 3.2.1 and 3.2.11). All cultures produced mycelia which darkened

Figure 3.1 Pigmented cultures of selected field isolates at 1 week

1. Isolate 517A/1. From decay of hoop pine, S.E. Queensland
2. Isolate 576B/1. From decay of hoop pine, S.E. Queensland
3. Isolate 1075/2. From root of hoop pine, S.E. Queensland
4. Isolate 1166A. From encrustation on hoop pine, S.E. Queensland
5. Isolate 1166B/4. From decay of hoop pine, S.E. Queensland
6. Isolate 1516. From basidiocarp on hoop pine, S.E. Queensland
7. Isolate 1518. From basidiocarp on hoop pine, S.E. Queensland
8. Isolate 2002. From basidiocarp on hoop pine, S.E. Queensland
9. Isolate 2261. From basidiocarp on hoop pine, N. Queensland
10. Isolate 2265. From basidiocarp on hoop pine, N. Queensland
11. Isolate LB4. From encrustation on rubber, Malaysia
12. Isolate LB5S. From basidiocarp on hoop pine, N. Queensland

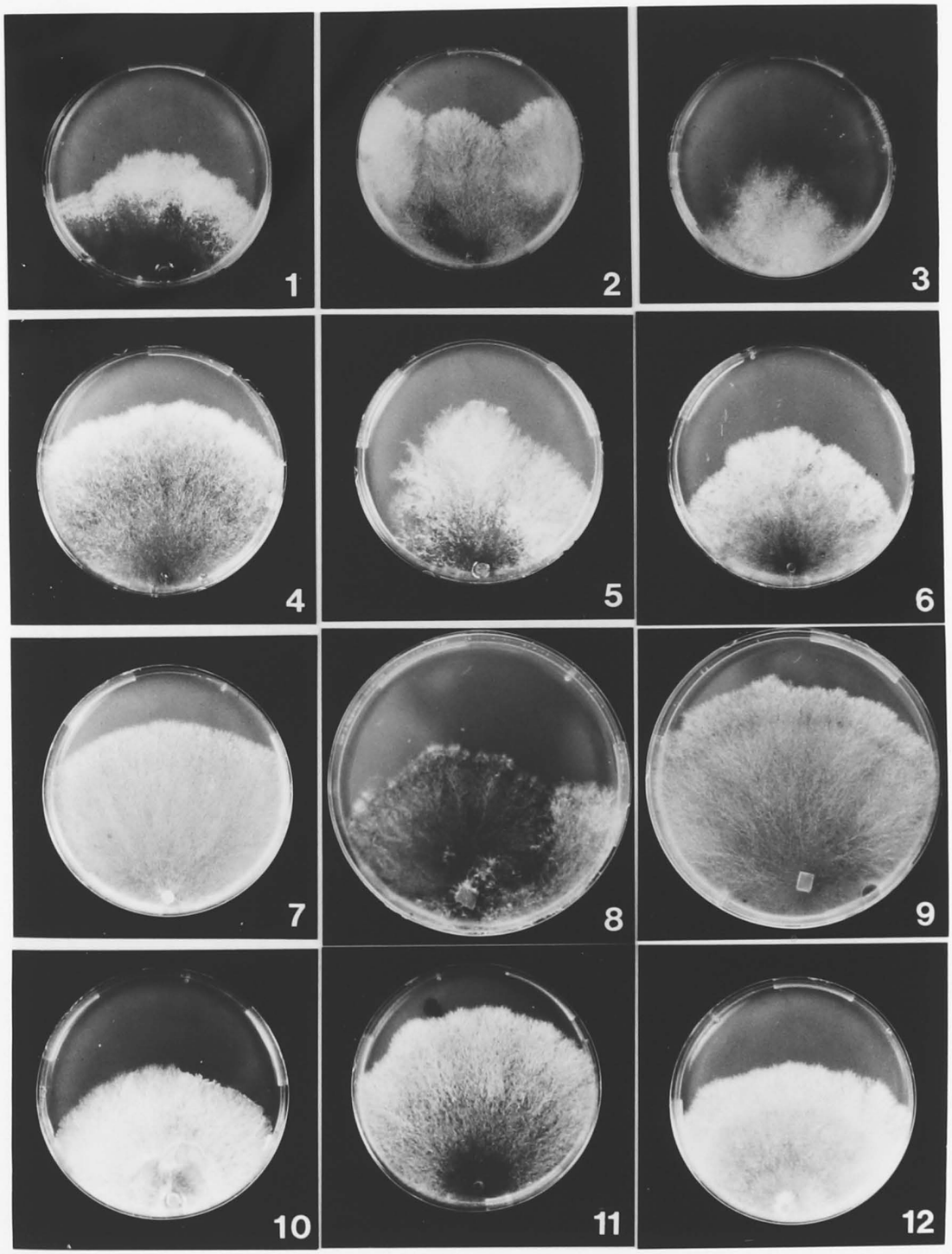
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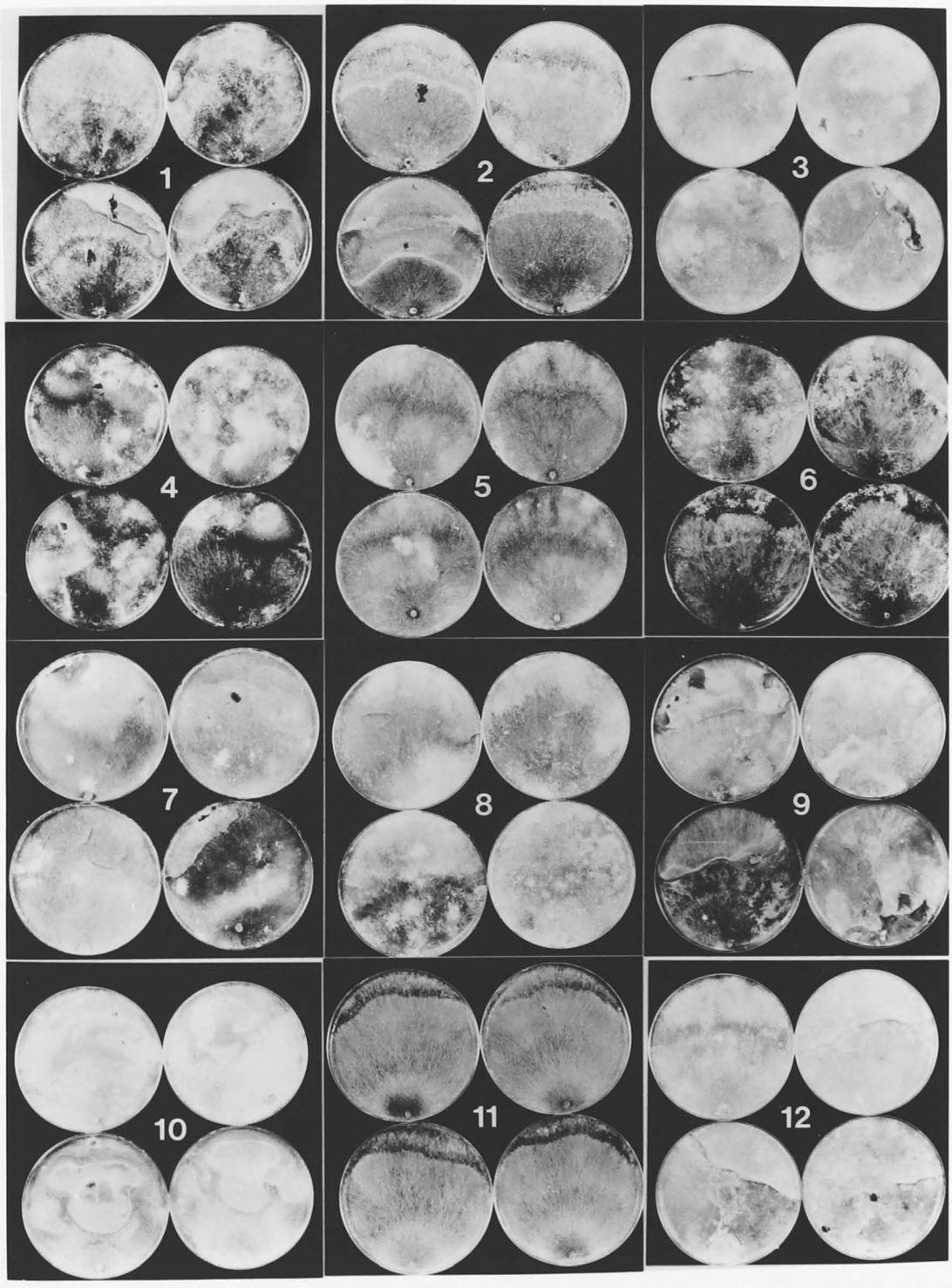
Figure 3.2 Pigmented cultures of selected field isolates at 6 weeks

1. Isolate 517A/1. From decay of hoop pine, S.E. Queensland
2. Isolate 576B/1. From decay of hoop pine, S.E. Queensland
3. Isolate 1075/2. From root of hoop pine, S.E. Queensland
4. Isolate 1166A. From encrustation on hoop pine, S.E. Queensland
5. Isolate 1166B/4. From decay of hoop pine, S.E. Queensland
6. Isolate 1516. From basidiocarp on hoop pine, S.E. Queensland
7. Isolate 1518. From basidiocarp on hoop pine, S.E. Queensland
8. Isolate 2002. From basidiocarp on hoop pine, S.E. Queensland
9. Isolate 2261. From basidiocarp on hoop pine, N.Queensland
10. Isolate 2265. From basidiocarp on hoop pine, N.Queensland
11. Isolate LB4. From encrustation on rubber, Malaysia
12. Isolate LB5S. From basidiocarp on hoop pine, N.Queensland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in aqueous KOH. However, such mycelia were always pigmented; mycelia from unpigmented areas of cultures were not affected by the reagent.

Crustose areas were developed randomly by many cultures. They were of two forms: (a) superficial, and (b) ribbon-like "zone lines" with one edge penetrating the substrate; and cultures produced either or both types. When first formed, crusts were Pale Luteous but rapidly turned Luteous, then Sienna, Umber, Bay, Rust and finally, Chestnut. Zone lines had bands of colour along their length, the latest developed were Pale Luteous, and the earliest, Chestnut. Colours displayed by mats of pigmented cultures are illustrated in Figure 3.3.

Pigmentation of the agar was consistently associated with the presence of pigmented mycelia. The agar remained unchanged beneath areas of mats which remained unpigmented. The intensity of the pigmentation increased with that of the mycelium above.

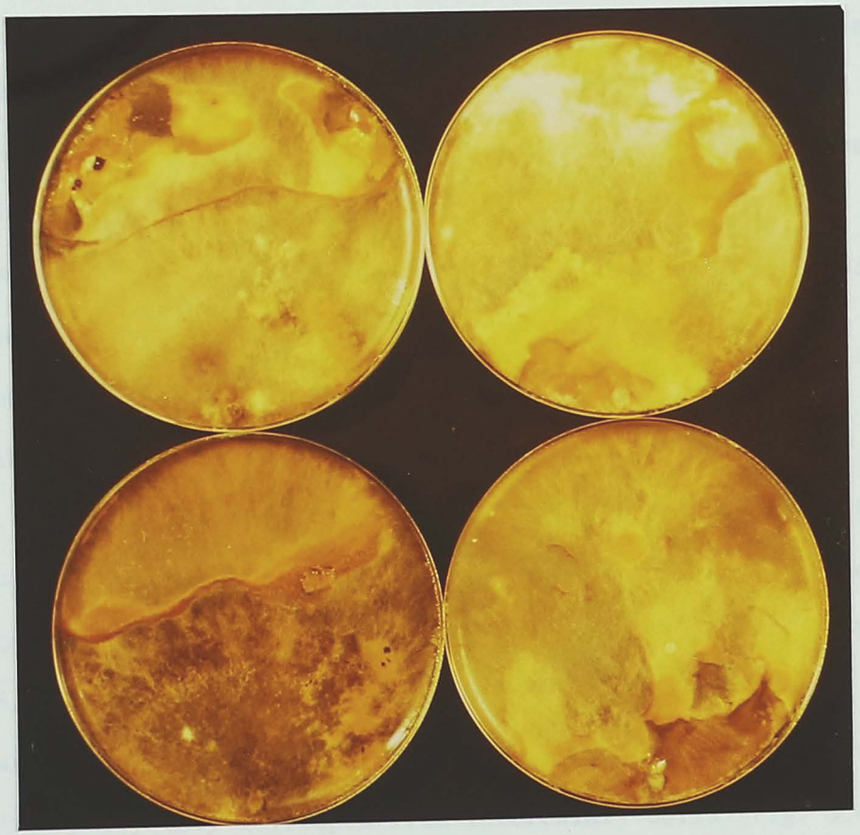
Oxidase reactions on GAA varied from strong to very strong, and those on TAA were consistently moderately strong. Radial growth of cultures after 7 days were 0-42 mm on GAA, and 22-42 mm on TAA. Tests with the alcoholic solution of guaiacum were negative on unpigmented areas of mats, and positive on pigmented areas. Colour reactions on the latter varied from Greyish Blue Green on undifferentiated mycelia to Dark Cyan Blue on crustose areas.

Even rudimentary basidiocarps were not produced by any culture by 6 weeks, and cultures had no noteworthy odour.

Microscopic Characters

Hyphae in the advancing zone were hyaline, septate and thin-walled and septal clamp connections were lacking (Fig. 3.4.1). Diameters ranged from 1.5 µm to 10.0 µm, but were mostly 2.0-5.0 µm. Many of the broader

Figure 3.3 Colours displayed at 6 weeks by mats of pigmented cultures of field isolate No. 2261



hyphae (8.0-10.0 µm) stained intensely with the cotton blue.

Aerial mycelia of older parts comprised both hyaline and pigmented hyphae. Morphology of the former was similar to that of hyphae in the advancing zone, except that narrower distal hyphae (1.5-5.0 µm) were frequently converted into arthrospores (the terminology of Talbot (1971) was adopted for these spores). Arthrospores were cylindrical with truncated or rounded ends, and were 2.5-30.0 µm long (Fig. 3.4.2).

Pigmented hyphae were pale to intense yellow-brown, septate and frequently branched, with thin to slightly thickened walls, and lacked clamp connections (Fig. 3.4.3). Septa were often of the dolipore type (Fig. 3.4.4). Hyphal diameters were 2.5-13.0 µm.

Four specialized structures, all modifications of pigmented hyphae, were recognized: fibre hyphae, staghorn hyphae, cuticular cells and interlocking hyphae.

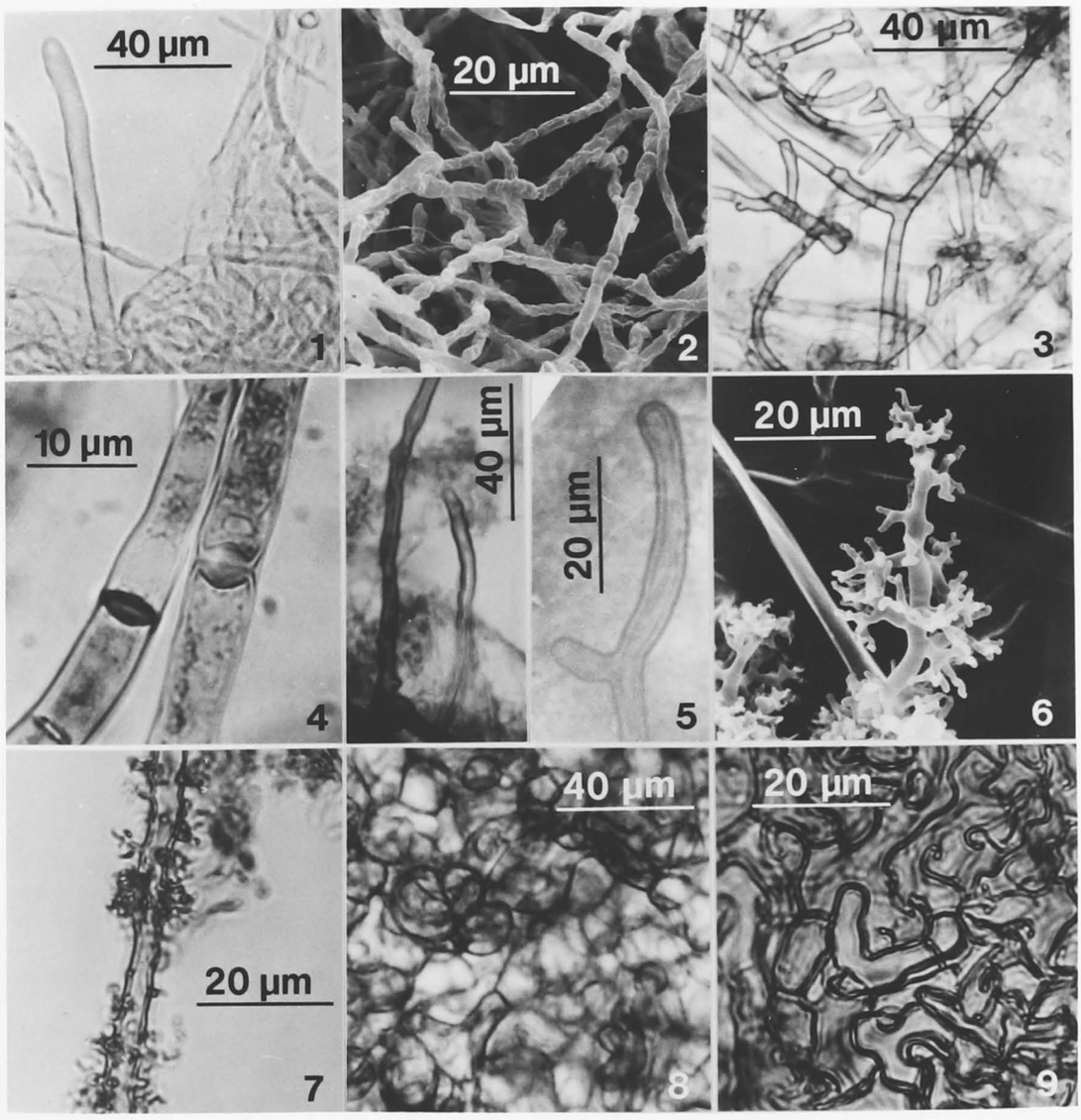
Fibre hyphae (3.0-5.5 µm diam) occurred as apical cells of pigmented hyphae. They were dark brown, branched, and had thick walls (Fig.3.4.5). They could be found anywhere in a culture, but usually in crustose areas.

Staghorn hyphae were dendritic, having a stem and lateral branches (Fig. 3.4.6). Branches divided dichotomously up to four times. The structures were usually modifications of apical cells of pigmented hyphae, but occasionally they developed from intercalary cells (Fig. 3.4.7). They had thick walls and were often septate (Fig. 3.4.7). Size varied but stem diameter at the base was mostly 2.5-4.0 µm and lengths of up to 50 µm were recorded. They occurred anywhere on a mat and were usually undetectable macroscopically. Occasionally, they developed in discrete patches which were Orange in colour and readily seen with the unaided eye.

The crusts formed by the fungus in culture were composed of cuticular

Figure 3.4 Microscopic characters of cultures

1. Hyphae at the advancing margin
2. Arthrospores
3. Undifferentiated pigmented hyphae
4. Dolipore septa
5. Fibre hyphae
6 - 7. Staghorn hyphae. These structures are usually modifications of apical cells of pigmented hyphae (Fig.6), but occasionally they may develop from intercalary cells (Fig. 7)
8. Cuticular cells
9. Interlocking hyphae


40 µm
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6
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7
40 µm
8
20 µm
9

cells, or interlocking hyphae, or both. Cuticular cells were approximately globose (4.0-36.0 µm diam) and had yellow-brown, thin to slightly thickened walls (Fig. 3.4.8). Interlocking hyphae were 8.0-12.0 µm diameter and septate and had yellow-brown to dark brown, thick walls (Fig. 3.4.9).

Only hyaline hyphae, unmodified pigmented hyphae and cuticular cells were found among submerged mycelia. Their respective morphologies were similar to those of aerial mycelia.

Key Code - 2.6.(8).10.11.35.37.39.42.54.55

Cultural Characters of the Unpigmented Type

Macroscopic Characters

Cultures grew rapidly, covering the medium in 8-14 days. Advancing margins were white, uneven, raised, cottony and 10-15 mm wide. The older mat was initially white and cottony to woolly but soon collapsed becoming white felty to woolly. Cultures did not produce crustose areas or pigmented mats, nor was the medium discoloured. Mycelia were unaffected by aqueous KOH. Basidiocarps were not formed, nor was any noteworthy odour produced. The appearances of cultures at 1 week and at 6 weeks are illustrated in Fig. 3.5.

Tests for extracellular oxidase were negative on GAA, and with alcoholic guaiacum. However, moderately strong reactions on TAA were recorded for all isolates. Radial growth of cultures after 7 days were 0-8 mm on GAA and 12-36 mm on TAA.

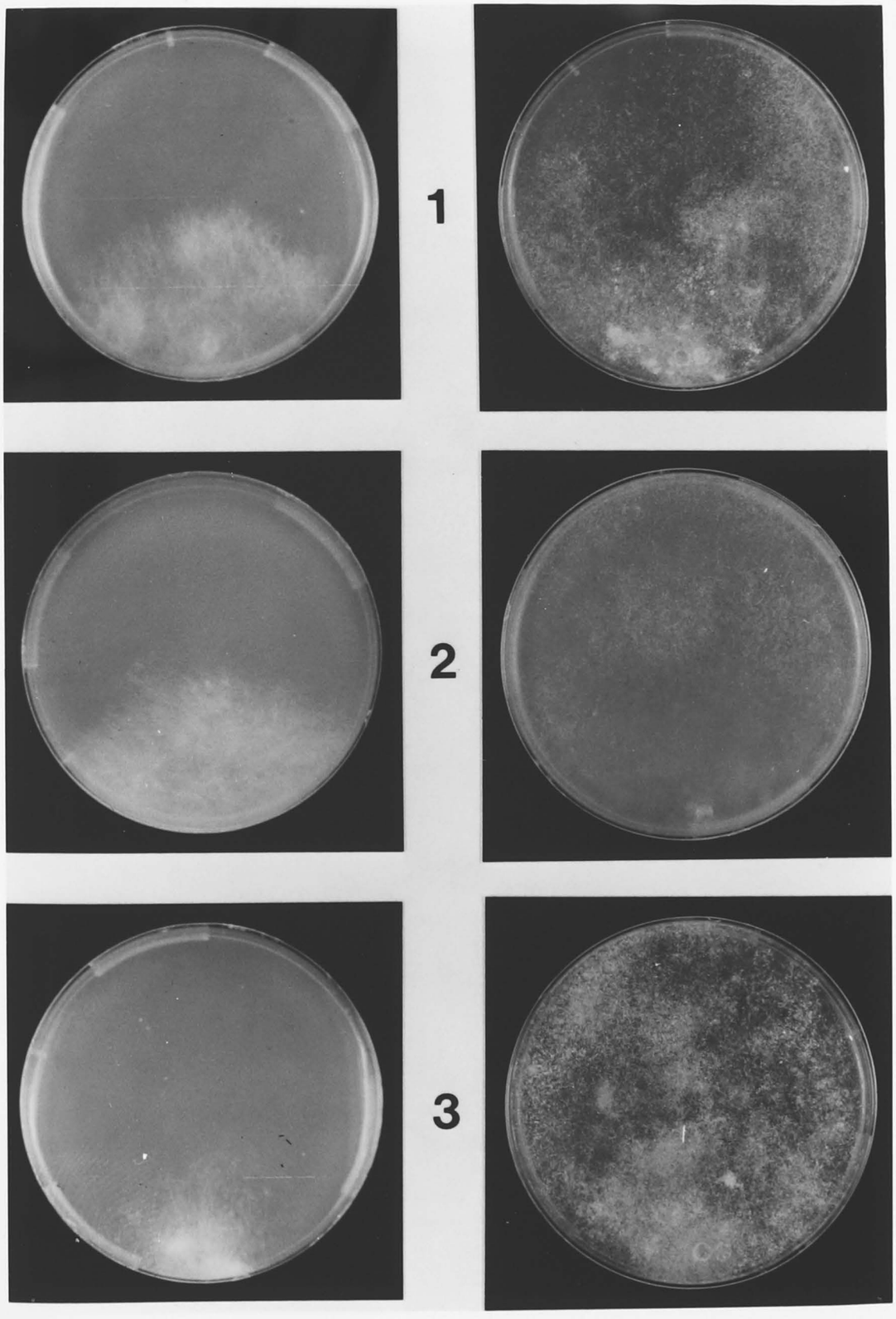
Microscopic Characters

The only significant microscopic features in aerial mycelia of

Figure 3.5 Unpigmented cultures of selected field isolates at 1 week, and at 6 weeks

1. Isolate 1516. From basidiocarp on hoop pine, S.E.Queensland
2. Isolate 2250. From basidiocarp on hoop pine, N. Queensland
3. Isolate LB3. From root of rubber, Malaysia

Cultures to the left were incubated for 7 days, and those to the right for 6 weeks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unpigmented cultures were hyaline hyphae and arthrospores. The morphologies of these features were similar to those of the pigmented cultures except that diameters were approximately 2.0 µm greater in unpigmented cultures. Submerged and aerial hyphae had a similar morphology.

Key Code - 2.6.7.35.36.38.42.54.55

3.2.2.2 Monoarthrospore Isolates

Macroscopic Characters

Growth rate of cultures, and the topographies, textures and colours of advancing margins and older mycelia were similar to those of the parent isolate (LB5S). However, the extent and intensity of mycelial pigmentation, and the area of mats covered by crust were generally less than those of the parent (Fig. 3.6). Basidiocarps were not formed and cultures had no noteworthy odour.

In tests for extracellular oxidase, all isolates gave moderately strong reactions on both GAA and TAA. Radial growth of 7 day old cultures was 1-13 mm on GAA and 18-30 mm on TAA. As for field isolates, positive results with alcoholic guaiacum were obtained only on pigmented mycelia.

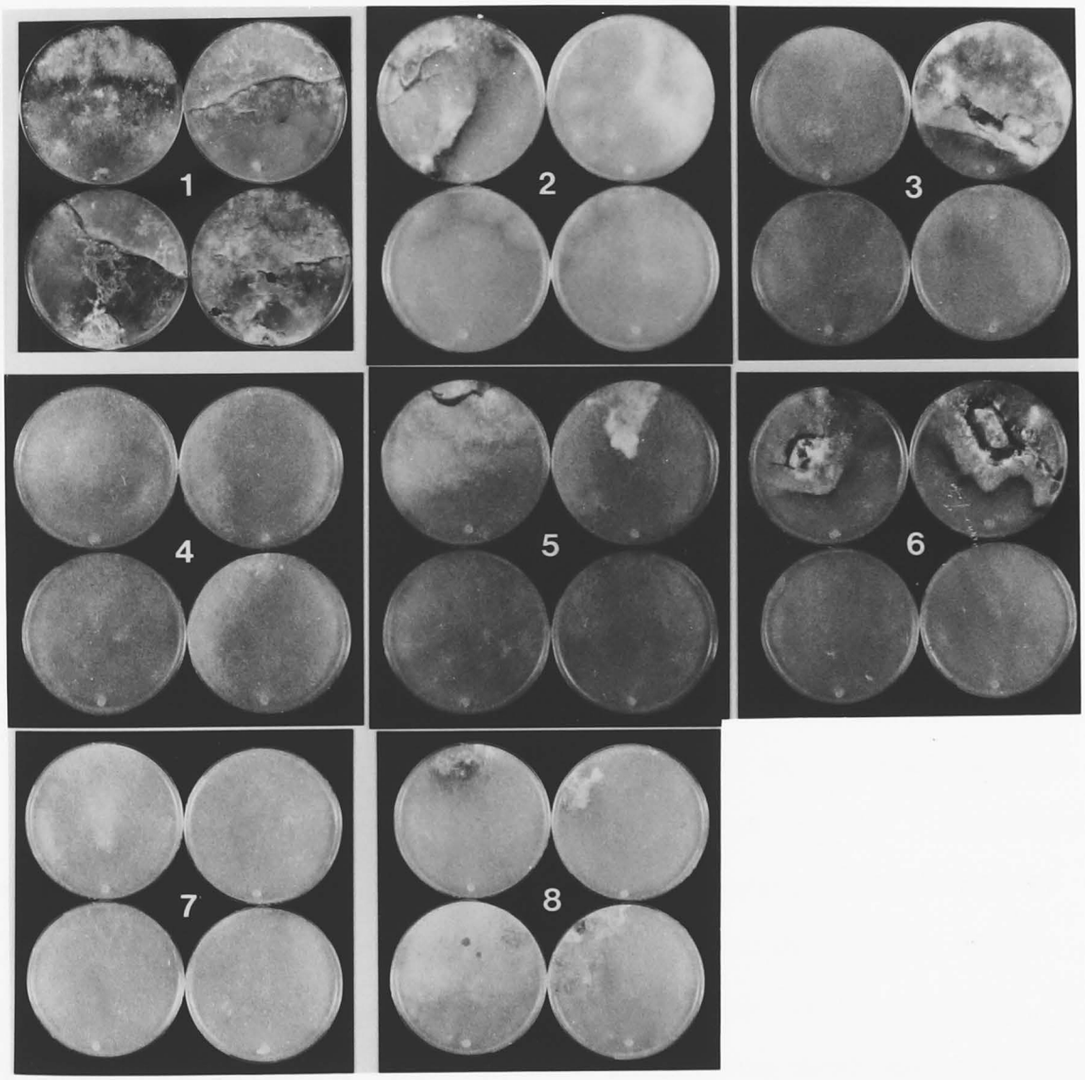
Microscopic Characters

All features produced by field isolates, except fibre hyphae, were found among the arthrospore isolates; but the parent isolate also lacked fibre hyphae.

Figure 3.6 Appearance of 6 week old cultures of selected monoarthrospore isolates

1. Parent isolate (LB5S)

2 - 8. Selected monoarthrospore isolates, covering the range of culture types encountered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3.2.2.3 Monobasidiospore Isolates

Macroscopic Characters

Growth rates of isolates varied from very slow (dishes not covered in 6 weeks) to rapid (dishes covered in 2 weeks). Advancing margins were white, uneven, raised, light cottony to woolly and 5-20 mm wide (Fig. 3.7).

Aerial mycelium behind the advancing margin was initially white and raised cottony to farinaceous woolly but it soon collapsed in some cultures to become appressed felty. The topography and texture of older mats, and the extent and intensity of mycelial pigmentation, varied considerably among isolates (Fig. 3.8). Three distinct cultural types, which could be separated by their topography and colour, were recognized: appressed and unpigmented (Fig. 3.8.6); appressed and intensely pigmented (Figs. 3.8.2, 3.8.8, 3.8.12) and raised with variable pigmentation (Figs. 3.8.3, 3.8.7, 3.8.11). The first two types displayed a form of cultural dimorphism in which spontaneous reversions from the one type to the other took place; sectoring often occurred in a culture and both types were then present (Figs. 3.8.4, 3.8.5, 3.8.9, 3.8.10). Dimorphism was not observed in the third cultural type. The extent and intensity of mycelial pigmentation in raised cultures varied from small, lightly pigmented areas in a predominantly unpigmented mat, to an extensive intensely pigmented mat with a bloom of unpigmented mycelium. The development of aerial mycelia in the raised cultures was usually less profuse than that in cultures of field isolates.

Mycelial crusts occurred in pigmented cultures, but were less frequent and less extensive than in cultures of the basidiocarp isolate (LB5S).

Figure 3.7 Cultures of selected monobasidiospore isolates at 1 week.

1. Isolate LB5S from context of the basidiocarp from which single spore isolations were made
2. Isolate B2
3. Isolate B4
4. Isolate B8
5. Isolate B9
6. Isolate B10
7. Isolate B15
8. Isolate B16
9. Isolate B19
10. Isolate B26
11. Isolate B28
12. Isolate B29

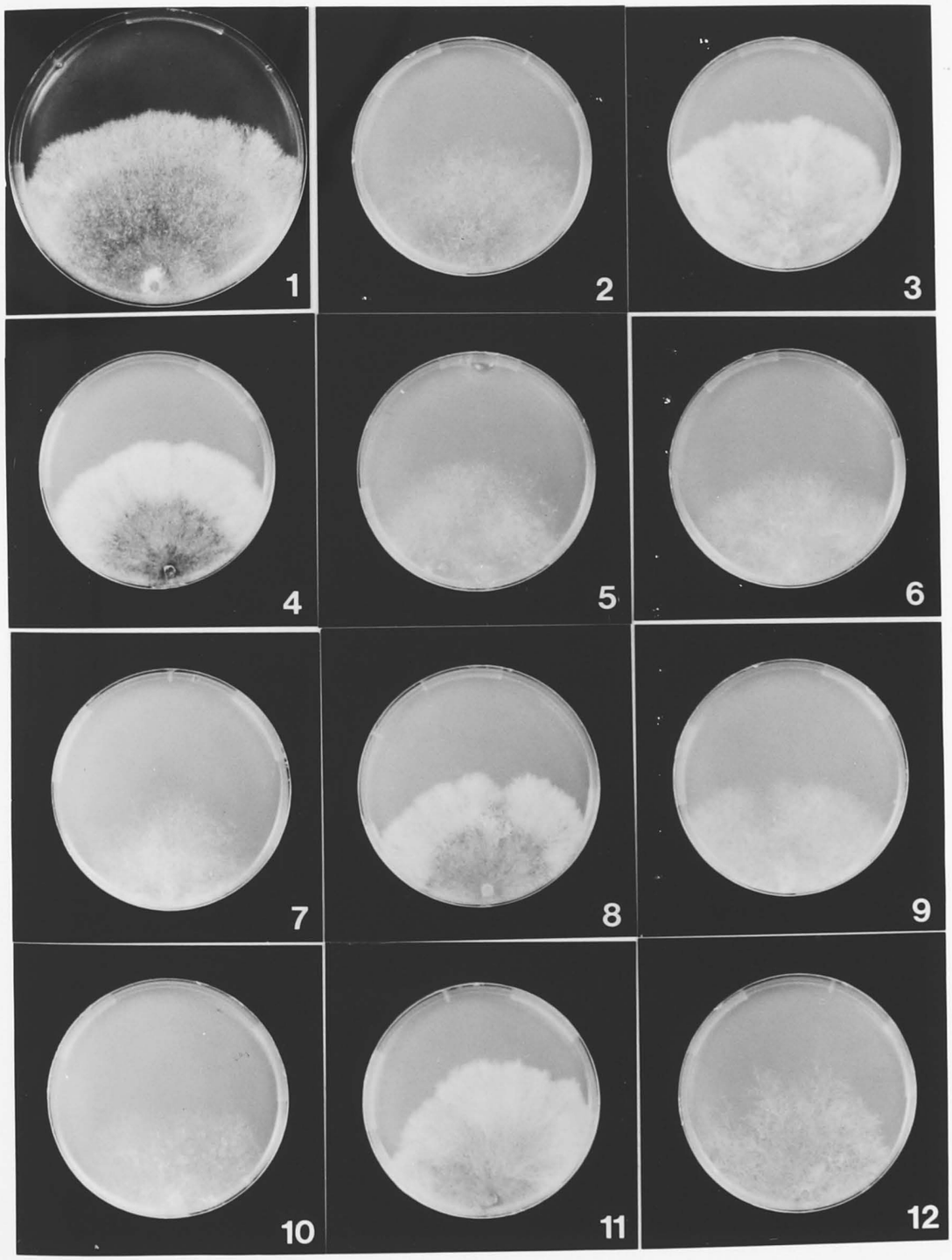
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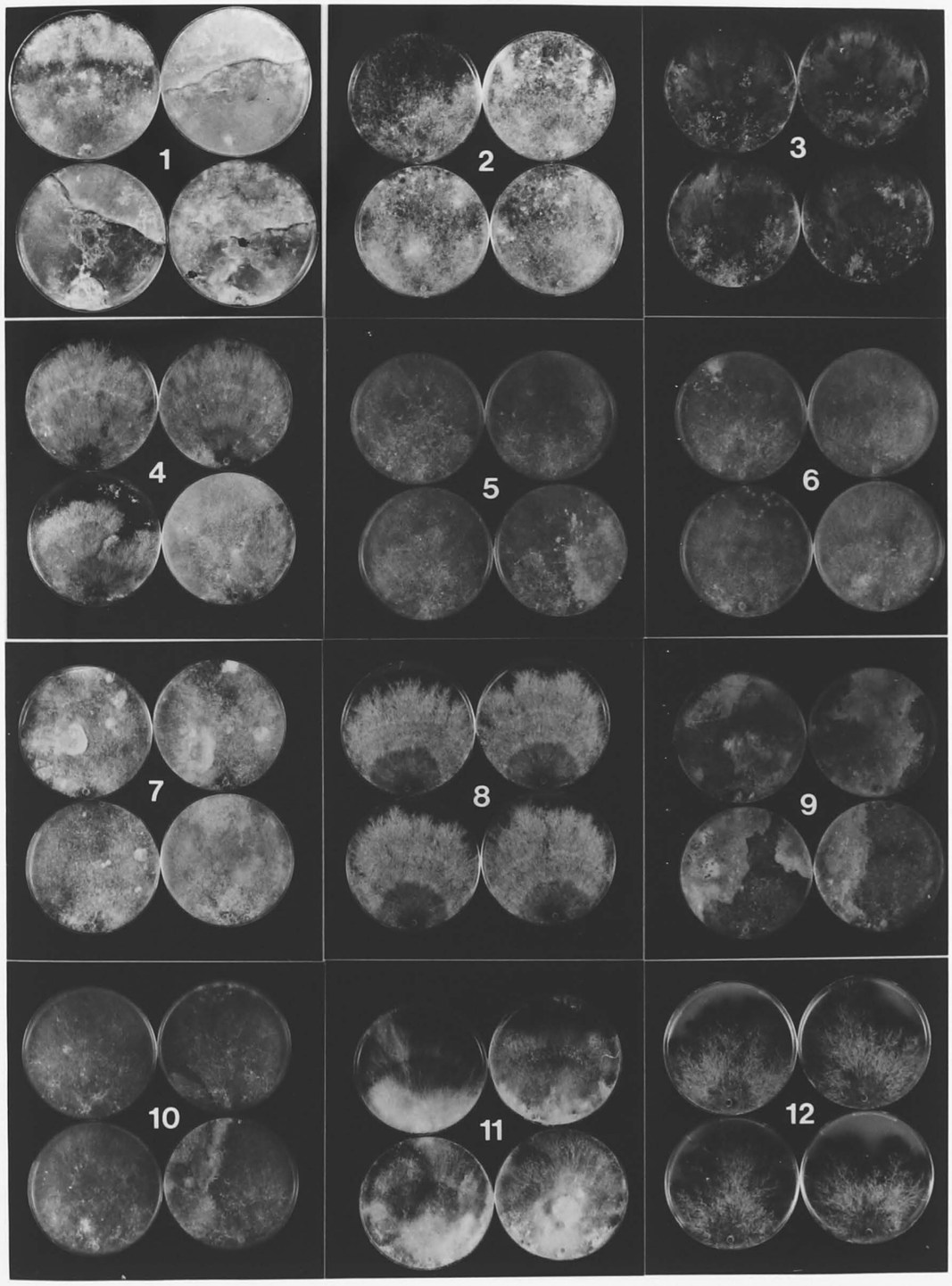
Figure 3.8 Cultures of selected monobasidiospore isolates at 6 weeks

1. Isolate LB5S from context of the basidiocarp from which single spore isolations were made
2. Isolate B2
3. Isolate B4
4. Isolate B8
5. Isolate B9
6. Isolate B10
7. Isolate B15
8. Isolate B16
9. Isolate B19
10. Isolate B26
11. Isolate B28
12. Isolate B29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Two different odours were detected among appressed cultures. Unpigmented cultures had an earthy or musty odour, whereas pigmented cultures had a distinctly fragrant odour. Raised cultures had no noteworthy odour.

Oxidase reactions on GAA varied from negative to very strong, and those on TAA were consistently moderately strong. Radial growth of 7 day old cultures was 0-38 mm on GAA and 18-42 mm on TAA. Positive results with alcoholic guaiacum were obtained only on pigmented mycelia.

Microscopic Characters

All features produced by field isolates, except fibre hyphae, were found among the basidiospore isolates. The parent isolate, however, also lacked fibre hyphae.

3.2.3 Discussion

All the cultural characters of *P. noxius* described by Bakshi *et al.* (1970) were observed in the present studies. In addition arthrospores, which were not recorded by those authors, were a regular feature of the fungus in these studies. Bakshi *et al.* (1970) considered that farinaceous felty, deep brown mats with crustose areas composed of cuticular cells and interlocking hyphae, and staghorn hyphae were diagnostic for *P. noxius*. Many Hymenomycetes have characters in common with *P. noxius* but relatively few have either staghorn hyphae or arthrospores. A review of literature on cultural characters of Hymenomycetes showed that only *Poria vincta* (Berk.)Cke. has both (Setliff, 1972). Surprisingly, other *Phellinus* spp. have neither (Campbell, 1938; Nobles, 1965; Bakshi *et al.*, 1970; Sen, 1973; Niemelä, 1974, 1975, 1977b). *Phellinus noxius* may

be separated from *P. vincta*, as cultures of the latter have mycelia with regular septal clamp connections in the advancing zone, encrusted staghorn hyphae, pink mats, and a marbelled appearance when viewed from the underside of dishes. From the present studies, the following characters might be considered diagnostic for *P. noxius*: brown mats with crustose areas of cuticular cells, or interlocking hyphae, or both; hyphae which lack septal clamp connections; staghorn hyphae and arthrospores.

The stable unpigmented culture type of *P. noxius* is a phenomenon which appears to be unusual, if not unique, among Hymenomycetes with pigmented cultures. Isolations of this culture type directly from field collections may be rare. Cultures from all Queensland collections were pigmented when newly isolated. Those of all Malaysian isolates were also pigmented when received. The Sri Lankan isolate (LB1), however, was unpigmented. The subculturing history of this isolate is unknown to the author but it was not a fresh isolation having been obtained from material collected in December, 1976. The pure stable unpigmented culture is possibly an artifact of culturing the fungus on a synthetic medium, and may not occur in nature. It is therefore considered to be a variant from the usual pigmented field isolate.

If such a culture was isolated from a field collection, the lack of pigmentation and specialized structures in the variant would seem to hinder the positive identification of the fungus in the laboratory. However, the lack of characters may be diagnostic. Of 149 species from 30 genera of Hymenomycetes described by Nobles (1965) and of 137 species from eleven genera of Indian polypores described by Bakshi *et al.* (1969, 1970) and Sen (1973), only *Peniophora gigantea* (Fr.) Massee has the same Key Code. The two can be separated as *P. gigantea* has occasional septal clamp connections on its hyphae (Nobles, 1965).

Although less pigmented, cultures of monoarthrospore isolates appeared to resemble more closely the parent isolate (LB5S) than most of the other field isolates (cf. Figs. 3.2 and 3.6). A check with a second field isolate (1141C) which differed from LB5S in cultural appearance, showed that its monoarthrospore isolates also closely resembled the parent. Comparisons between these results and those for other Hymenomycetes were not possible as reports of similar studies were not found in the literature.

Distinct cultural types among monobasidiospore isolates also occur in *Heterobasidion annosum* (Bega & Hendrix, 1962) and *Lenzites trabea* (Fr.) Pers. (Bezemer, 1973), but the dimorphism observed in *P. noxius* has not been reported for other Hymenomycetes. Cultures of field isolates of *Phellinus nigricans*(Fr.)Karst., *Phellinus populicola* Niemelä, and *Phellinus tremulae* exhibit dimorphism (Niemelä, 1974, 1975), so monobasidiospore isolates of these species might show this phenomenon also.

3.3 EXPERIMENT 2: VARIATION IN OCCURRENCE OF CULTURAL CHARACTERS

3.3.1 Materials and Methods

Data for this experiment were obtained from cultures prepared for Experiment 1 (Section 3.2.1). Fungal contamination reduced the number of replicate MEA cultures of many isolates from six to four; hence in each study four uncontaminated cultures were selected at random from those available, and the characters in each were recorded. Records of occurrences of obvious crusts, mat pigmentation, changes in the colour of the medium and all microscopic characters were taken from 6 week old cultures. Where appropriate, characters were expressed in terms of Nobles' Code Symbols (Nobles, 1965) for compilation of results in tabular form.

Statistical comparisons of isolates were not attempted because of the inherent unreliability of the data: some microscopic characters and very small areas of crust could be overlooked in some cultures.

3.3.2 Results

3.3.2.1 Field Isolates

Pigmented Isolates

Results are presented in Table 3.1. Characters consistently present in all MEA cultures were growth rate (Code Symbol 42), pigmented mats (37), fungal-induced brown coloration of the medium (39), clamp-less hyphae (6) and arthrospores (35). Also consistent were the intensity of reactions for extracellular oxidase on TAA (moderately strong) and positive results for oxidase with alcoholic guaiacum. The occurrence of all other characters usually varied among isolates, studies, and cultures within the one study.

Reactions for oxidase on GAA varied from moderately strong (+++) to very strong (+++++). Some isolates (1075/2, 1166B/4, 1354B, LB5S) gave moderately strong reactions in all tests; and others (1516, 1518, LB3), strong (++++) to very strong reactions. One isolate (LB2) gave different reactions in all three studies, covering the range recorded for all other pigmented isolates. Radial growth of 7 day old cultures of the one isolate usually varied widely between studies, the extreme example being demonstrated by LB2 which grew 0-42 mm in different studies. Growth did not occur where very strong reactions were recorded, an observation consistent with those of Davidson *et al.* (1938).

Obvious crusts formed regularly in cultures of some isolates (1075/2, 1166B/4, LB3, LB5W), and infrequently in others (1141C, 1167, 1516, LB4).

Table 3.1 Macroscopic and microscopic characters recorded in pigmented cultures of field isolates

Isolate No.	Study No.	Macroscopic characters: Extracellular oxidase reactions[a] GAA	TAA	Gum Guaiacol	Obvious crusts	Microscopic characters: Noble's Code Symbols[b] 36	37	38	39	42	6	8	10	11	35	Staghorn hyphae
517A/1	1	++++15	+++22	+			4[c]		4[c]	4[c]	4[c]				4[c]	4[c]
	2	+++30	+++33	+	2[c]		4[c]		4[c]	4[c]	4[c]			2[c]	4[c]	3[c]
	3	+++35	+++42	+	3[c]		4[c]		4[c]	4[c]	4[c]			3[c]	4[c]	4[c]
586B/1	1	++++25	+++41	+	2[c]		4[c]		4[c]	4[c]	4[c]		2[c]	2[c]	4[c]	3[c]
	2	+++35	+++42	+			4[c]		4[c]	4[c]	4[c]				4[c]	
	3	+++31	+++42	+	1[c]		4[c]		4[c]	4[c]	4[c]			1[c]	4[c]	4[c]
1075/2	1	+++33	+++34	+	4[c]		4[c]		4[c]	4[c]	4[c]		3[c]	4[c]	4[c]	
	2	+++30	+++42	+	4[c]		4[c]		4[c]	4[c]	4[c]			4[c]	4[c]	4[c]
	3	+++37	+++39	+	4[c]		4[c]		4[c]	4[c]	4[c]	2[c]		4[c]	4[c]	3
1141C	1	+++42	+++42	+			4[c]		4[c]	4[c]	4[c]				4[c]	4[c]
	2	++++25	+++42	+			4[c]		4[c]	4[c]	4[c]				4[c]	4[c]
	3	+++33	+++42	+	2[c]		4[c]		4[c]	4[c]	4[c]			2[c]	4[c]	4[c]
1166A	1	++++17	+++33	+	4[c]		4[c]		4[c]	4[c]	4[c]	1[c]	3[c]	4[c]	4[c]	3[c]
	2	+++27	+++42	+			4[c]		4[c]	4[c]	4[c]				4[c]	
	3	++++23	+++42	+	4[c]		4[c]		4[c]	4[c]	4[c]			4[c]	4[c]	4[c]
1166B/4	1	+++10	+++23	+	4[c]		4[c]		4[c]	4[c]	4[c]	2[c]	4[c]	4[c]	4[c]	4[c]
	2	+++19	+++30	+	4[c]		4[c]		4[c]	4[c]	4[c]			4[c]	4[c]	
	3	+++27	+++38	+	4[c]		4[c]		4[c]	4[c]	4[c]			2[c]	2[c]	4[c]
1167	1	++++16	+++42	+	2[c]		4[c]		4[c]	4[c]	4[c]	1[c]	2[c]	1[c]	4[c]	4[c]
	2	++++24	+++42	+			4[c]		4[c]	4[c]					4[c]	4[c]
	3	+++25	+++42	+			4[c]		4[c]	4[c]	4[c]				4[c]	4[c]
1354B	1	+++26	+++42	+	3[c]		4[c]		4[c]	4[c]	4[c]		2[c]	3[c]	4[c]	4[c]
	2	+++22	+++42	+	4[c]		4[c]		4[c]	4[c]	4[c]		4[c]	4[c]	4[c]	4[c]
	3	+++24	+++42	+	2[c]		4[c]		4[c]	4[c]	4[c]			2[c]	4[c]	4[c]
1516	1	++++33	+++32	+	1[c]		4[c]		4[c]	4[c]	4[c]		1[c]		4[c]	4[c]
	2	++++30	+++40	+			4[c]		4[c]	4[c]	4[c]				4[c]	4[c]
	3	++++17	+++35	+			4[c]		4[c]	4[c]	4[c]				4[c]	4[c]
1518	1	+++++ 0	+++28	+	4[c]		4[c]		4[c]	4[c]	4[c]		2[c]	4[c]	4[c]	4[c]
	2	++++17	+++42	+	3[c]		4[c]		4[c]	4[c]	4[c]		3[c]	3[c]	4[c]	
	3	++++24	+++42	+	4[c]		4[c]		4[c]	4[c]	4[c]		3[c]	4[c]	4[c]	4[c]
2002	1	+++23	+++25	+			4[c]		4[c]	4[c]	4[c]				4[c]	
	2	++++40	+++42	+	3[c]		4[c]		4[c]	4[c]	4[c]		3[c]	3[c]	4[c]	3[c]
	3	++++37	+++42	+	2[c]		4[c]		4[c]	4[c]	4[c]		2[c]	2[c]	4[c]	
2250	1	++++11	+++36	+			4[c]		4[c]	4[c]	4[c]				4[c]	1[c]
	2	+++15	+++37	+	2[c]		4[c]		4[c]	4[c]	4[c]	1[c]		2[c]	4[c]	2[c]
	3	+++13	+++36	+	3[c]		4[c]		4[c]	4[c]	4[c]		3[c]	3[c]	4[c]	3[c]
2261	1	++++38	+++38	+			4[c]		4[c]	4[c]	4[c]				4[c]	
	2	+++36	+++42	+	3[c]		4[c]		4[c]	4[c]	4[c]			3[c]	4[c]	3[c]
	3	+++39	+++40	+	4[c]		4[c]		4[c]	4[c]	4[c]		4[c]	4[c]	4[c]	4[c]
LB2	1	+++++ 0	+++42	+	4[c]		4[c]		4[c]	4[c]	4[c]		2[c]	4[c]	4[c]	4[c]
	2	++++33	+++42	+	3[c]		4[c]		4[c]	4[c]	4[c]		3[c]		4[c]	2[c]
	3	+++42	+++42	+	4[c]		4[c]		4[c]	4[c]	4[c]		2[c]	4[c]	4[c]	4[c]
LB3	1	+++++ 0	+++42	+	4[c]		4[c]		4[c]	4[c]	4[c]		4[c]	4[c]	4[c]	4[c]
	2	++++18	+++42	+	4[c]		4[c]		4[c]	4[c]	4[c]		4[c]	4[c]	4[c]	4[c]
	3	++++16	+++38	+	4[c]		4[c]		4[c]	4[c]	4[c]		4[c]	4[c]	4[c]	4[c]
LB4	1	+++++ 0	+++42	+			4[c]		4[c]	4[c]	4[c]				4[c]	
	2	+++39	+++42	+			4[c]		4[c]	4[c]	4[c]				4[c]	
	3	++++28	+++42	+	2[c]		4[c]		4[c]	4[c]	4[c]			2[c]	4[c]	3[c]
LB5S	1	+++31	+++37	+			4[c]		4[c]	4[c]	4[c]				4[c]	4[c]
	2	+++25	+++42	+			4[c]		4[c]	4[c]	4[c]				4[c]	4[c]
	3	+++42	+++42	+	3[c]		4[c]		4[c]	4[c]	4[c]			2[c]	3[c]	4[c]	4[c]
LB5W	1	+++31	+++37	+	4[c]		4[c]		4[c]	4[c]	4[c]		4[c]	4[c]	4[c]	4[c]
	2	++++41	+++42	+	4[c]		4[c]		4[c]	4[c]	4[c]		4[c]	4[c]	4[c]	
	3	+++27	+++42	+	4[c]		4[c]		4[c]	4[c]	4[c]		4[c]	4[c]	4[c]	4[c]

[a] Oxidase Reactions. GAA and TAA -, negative; +, very weak; ++, weak; +++, moderately strong; ++++, strong; +++++, very strong. Descriptions of these reactions are given in Appendix 6. Numbers represent radial growth (mm) in 7 days. Guaiacol: -, negative; +, positive.

[b] Code Symbols (from Nobles, 1965). Macroscopic Characters: 36, white or pale mats; 37, mats yellow or brown, at least in part; 38, agar unchanged in colour; 39, agar brown at least in part; 42, plates covered in 2 weeks; 43, plates covered in 3 weeks; 47, plates not covered in 6 weeks; 50, fragrant odour; 51, earthy or musty odour. Microscopic Characters: 6, hyphae lacking clamp connections; 8, fibre hyphae; 10, cuticular cells; 11, interlocking hyphae; 35, arthrospores.

[c] Number of cultures with this character. Where number is absent no culture had this character.

Crust formation by the remaining isolates was more variable. Crusts were usually composed of both cuticular cells (Code Symbol 10) and interlocking hyphae (11), but occasionally, only one of these structures was recorded in a culture.

Fibre hyphae (Code Symbol 8) were recorded in cultures of only five isolates. They were recorded in one culture only of each of three isolates (1166A, 1167, 2250); and in two cultures each of the other two isolates (1075/2, 1166B/4) in a single study.

Staghorn hyphae formed in all cultures of some isolates (1141C, 1167, 1354B, 1516, LB3, LB5S), but usually records of their occurrence in cultures of the one isolate varied within the one study or between studies.

Because of the wide variation in the occurrence of certain characters in cultures of pigmented field isolates of *P. noxius*, characters displayed in a single study of an isolate might not be definitive for the fungus. A summary of data from these studies (Table 3.2) shows that observations on an isolate might have to be repeated on at least three different occasions before all the features considered to be diagnostic for the fungus - brown mats (Code Symbol 37) with crustose areas of cuticular cells (10), or interlocking hyphae (11), or both; hyphae which lack clamp connections (6); arthrospores (35); and staghorn hyphae (see Section 3.2.3) are recorded.

Unpigmented Variants

Cultures of all variants were remarkably uniform in characters recorded on MEA, and in results of tests for extracellular oxidase on GAA and TAA (Table 3.3). All overgrew their dishes within 2 weeks, lacked mycelial pigmentation, induced no colour changes in MEA, and gave negative results

Table 3.2 Percentage of pigmented field isolates in each study, and in groupings of studies, recorded to have the various macroscopic and microscopic characters observed in cultures of *P. noxius*

Grouping	Macroscopic Characters										Microscopic Features					
	Extracellular Oxidase Reactions[a]			Obvious crusts[c]	Nobles' Code Symbols[b]											
	GAA	TAA	Gum Guaiacol		36	37	38	39	42	6	8	10	11	10or11	35	Staghorn hyphae
Study 1	100	100	100	62		100		100	100	100	17	62	56	62	100	78
Study 2	100	100	100	62		100		100	100	100	6	34	56	62	100	67
Study 3	100	100	100	90		100		100	100	100	11	56	90	90	100	90
Studies 1+2	100	100	100	54		100		100	100	100	22	67	78	84	100	95
Studies 1+3	100	100	100	100		100		100	100	100	22	90	95	100	100	95
Studies 2+3	100	100	100	90		100		100	100	100	11	62	90	90	100	95
Studies 1+2+3	100	100	100	100		100		100	100	100	28	90	95	100	100	100

[a] Percentage of positive reactions

[b] Code Symbols (from Nobles, 1965). Macroscopic Characters: 36, white or pale mats; 37, mats yellow or brown, at least in part; 38, agar unchanged in colour; 39, agar brown at least in part; 42, plates covered in 2 weeks. Microscopic Characters: 6, hyphae lacking clamp connections; 8, fibre hyphae; 10, cuticular cells; 11, interlocking hyphae; 35, arthrospores.

Table 3.3 Macroscopic and microscopic characters recorded in cultures of unpigmented variants of field isolates

Isolate No.	Study No.	Macroscopic characters: Extracellular oxidase reactions[a] GAA	TAA	Gum Guaiacol	Obvious crusts[c]	Microscopic characters: Noble's Code Symbols[b] 36	37	38	39	42	6	8	10	11	35	Staghorn hyphae
	1	- 8	+++ 16	-		4[c]		4[c]		4[c]	4[c]				4[c]	
517A/1	2	- 6	+++ 18	-		4[c]		4[c]		4[c]	4[c]				4[c]	
	3	- 7	+++ 15	-		4[c]		4[c]		4[c]	4[c]				4[c]	
	1	- 8	+++ 16	-		4[c]		4[c]		4[c]	4[c]				4[c]	
604/2	2	- 7	+++ 21	-		4[c]		4[c]		4[c]	4[c]				4[c]	
	3	- 7	+++ 22	-		4[c]		4[c]		4[c]	4[c]				4[c]	
	1	- 5	+++ 36	-		4[c]		4[c]		4[c]	4[c]				4[c]	
1075/2	2	- 5	+++ 30	-		4[c]		4[c]		4[c]	4[c]				4[c]	
	3	- 6	+++ 29	-		4[c]		4[c]		4[c]	4[c]				4[c]	
	1	- 2	+++ 15	-		4[c]		4[c]		4[c]	4[c]				4[c]	
1354B	2	- 1	+++ 19	-		4[c]		4[c]		4[c]	4[c]				4[c]	
	3	- 1	+++ 16	-		4[c]		4[c]		4[c]	4[c]				4[c]	
	1	- 2	+++ 29	-		4[c]		4[c]		4[c]	4[c]				4[c]	
1516	2	- 4	+++ 23	-		4[c]		4[c]		4[c]	4[c]				4[c]	
	3	- 1	+++ 30	-		4[c]		4[c]		4[c]	4[c]				4[c]	
	1	- 0	+++ 33	-		4[c]		4[c]		4[c]	4[c]				4[c]	
2250	2	- 0	+++ 36	-		4[c]		4[c]		4[c]	4[c]				4[c]	
	3	- 7	+++ 34	-		4[c]		4[c]		4[c]	4[c]				4[c]	
	1	- 3	+++ 24	-		4[c]		4[c]		4[c]	4[c]				4[c]	
2261	2	- 0	+++ 30	-		4[c]		4[c]		4[c]	4[c]				4[c]	
	3	- 4	+++ 25	-		4[c]		4[c]		4[c]	4[c]				4[c]	
	1	- 1	+++ 18	-		4[c]		4[c]		4[c]	4[c]				4[c]	
LB1	2	- 2	+++ 18	-		4[c]		4[c]		4[c]	4[c]				4[c]	
	3	- 1	+++ 19	-		4[c]		4[c]		4[c]	4[c]				4[c]	
	1	- 0	+++ 14	-		4[c]		4[c]		4[c]	4[c]				4[c]	
LB3	2	- 0	+++ 13	-		4[c]		4[c]		4[c]	4[c]				4[c]	
	3	- 0	+++ 12	-		4[c]		4[c]		4[c]	4[c]				4[c]	

[a] Oxidase Reactions. GAA and TAA -, negative; +, very weak; ++, weak; +++, moderately strong; ++++, strong; +++++, very strong. Descriptions of these reactions are given in Appendix 6. Numbers represent radial growth (mm) in 7 days. Guaiacol: -, negative; +, positive.

[b] Code Symbols (from Nobles, 1965). Macroscopic Characters: 36, white or pale mats; 37, mats yellow or brown, at least in part; 38, agar unchanged in colour; 39, agar brown at least in part; 42, plates covered in 2 weeks; 43, plates covered in 3 weeks; 47, plates not covered in 6 weeks; 50, fragrant odour; 51, earthy or musty odour. Microscopic Characters: 6, hyphae lacking clamp connections; 8, fibre hyphae; 10, cuticular cells; 11, interlocking hyphae; 35, arthrospores.

[c] Number of cultures with this character. Where number is absent no culture had this character.

in tests for oxidase with alcoholic guaiacum. The only microscopic characters observed were thin-walled, clampless, unmodified hyphae, and arthrospores. All variants gave negative oxidase reactions on GAA and moderately strong reactions on TAA.

Most individual variants produced similar radial growth after 7 days on GAA or TAA, in all three studies; but growth on the latter was much greater. Differences occurred between variants on both media. Variant LB3 did not grow on GAA, whereas 517A/1 and 604/2 grew 6-8 mm; and LB3 grew 12-14 mm on TAA and 2250, 33-36 mm.

3.3.2.2 Monoarthrospore Isolates

Results are presented in Table 3.4. All isolates gave moderately strong oxidase reactions on both GAA and TAA. On GAA, radial growth of all but one isolate was within the narrow range 8-13 mm (the exception grew only 1 mm), and on TAA, was within the range 18-30 mm. Positive reactions with alcoholic guaiacum were recorded only on pigmented mats.

Eleven isolates produced crusts, but only one formed them in all four cultures. At least one culture of nineteen isolates and all cultures of three isolates, remained unpigmented. Only cultures with pigmented mats induced a brown coloration in the medium.

Nineteen isolates overgrew their dishes in 2 weeks, the remaining isolate in 3 weeks.

Hyphae of all cultures lacked septal clamp connections, and bore chains of arthrospores. Fibre hyphae were not observed in any culture. Crusts contained only interlocking hyphae, or both interlocking hyphae and cuticular cells. Staghorn hyphae were observed in cultures of only six isolates, but not in all four cultures of the one isolate.

Table 3.4 Macroscopic and microscopic characters recorded in cultures of monoarthrospore isolations from the one parent field isolate (LB5S)

Isolate No.	Macroscopic Characters											Microscopic characters					
	Extracellular oxidase reactions[a]			Obvious crusts	Noble's Code Symbols[b]												Staghorn hyphae
	GAA	TAA	Gum Guaiacol		36	37	38	39	42	43	6	8	10	11	35		
A 1	+++ 13	+++ 24	+	3[c]		4[c]		4[c]	4[c]		4[c]		1[c]	3[c]	4[c]		
A 2	+++ 8	+++ 18	+		2[c]	2[c]	2[c]	2[c]	4[c]		4[c]				4[c]		
A 3	+++ 9	+++ 25	+	1[c]	2[c]	2[c]	2[c]	2[c]	4[c]		4[c]		1[c]	1[c]	4[c]		
A 4	+++ 11	+++ 25	+	4[c]		4[c]		4[c]	4[c]		4[c]		3[c]	4[c]	4[c]		3[c]
A 5	+++ 12	+++ 26	+	2[c]	1[c]	3[c]	1[c]	3[c]	4[c]		4[c]		1[c]	2[c]	4[c]		2[c]
A 6	+++ 10	+++ 27	-		4[c]		4[c]		4[c]		4[c]				4[c]		
A 7	+++ 10	+++ 23	+	1[c]	2[c]	2[c]	3[c]	1[c]	4[c]		4[c]		1[c]	1[c]	4[c]		
A 8	+++ 8	+++ 21	+	1[c]	2[c]	2[c]	3[c]	1[c]	4[c]		4[c]		1[c]	1[c]	4[c]		1[c]
A 9	+++ 1	+++ 26	+		3[c]	1[c]	4[c]		4[c]		4[c]				4[c]		
A10	+++ 10	+++ 27	+	1[c]	2[c]	2[c]	2[c]	2[c]	4[c]		4[c]			1[c]	4[c]		
A11	+++ 12	+++ 28	-	2[c]	2[c]	2[c]	2[c]	2[c]	4[c]		4[c]			2[c]	4[c]		2[c]
A12	+++ 13	+++ 25	+		4[c]		4[c]		4[c]		4[c]				4[c]		
A13	+++ 10	+++ 25	+		3[c]	1[c]	3[c]	1[c]	4[c]		4[c]				4[c]		
A14	+++ 13	+++ 26	+		3[c]	1[c]	4[c]		4[c]		4[c]				4[c]		
A15	+++ 11	+++ 27	+	1[c]		4[c]		4[c]	4[c]		4[c]		1[c]	1[c]	4[c]		1[c]
A16	+++ 12	+++ 27	+	2[c]	1[c]	3[c]	1[c]	3[c]		4[c]	4[c]			2[c]	4[c]		2[c]
A17	+++ 11	+++ 25	-		4[c]		4[c]		4[c]		4[c]				4[c]		
A18	+++ 13	+++ 28	+			4[c]		4[c]	4[c]		4[c]				4[c]		
A19	+++ 13	+++ 26	+		3[c]	1[c]	4[c]		4[c]		4[c]				4[c]		
A20	+++ 13	+++ 30	+	2[c]	1[c]	3[c]	2[c]	2[c]	4[c]		4[c]			2[c]	4[c]		

[a] Oxidase Reactions. GAA and TAA -, negative; +, very weak; ++, weak; +++, moderately strong; ++++, strong; +++++, very strong. Descriptions of these reactions are given in Appendix 6. Numbers represent radial growth (mm) in 7 days. Guaiacol: -, negative; +, positive.

[b] Code symbols (from Nobles, 1965). Macroscopic Characters: 36, white or pale mats; 37, mats yellow or brown, at least in part; 38, agar unchanged in colour; 39, agar brown at least in part; 42, plates covered in 2 weeks; 43, plates covered in 3 weeks; 47, plates not covered in 6 weeks; 50, fragrant odour; 51, earthy or musty odour. Microscopic Characters: 6, hyphae lacking clamp connections; 8, fibre hyphae; 10, cuticular cells; 11, interlocking hyphae; 35, arthrospores.

[c] Number of cultures with this character. Where number is absent no culture had this character.

3.3.2.3 Monobasidiospore Isolates

The occurrence of most characters varied widely among isolates (Table 3.5). Characters which occurred regularly in all isolates were moderately strong oxidase reactions on TAA, hyphae which lacked clamp connections (Code Symbol 6), and arthrospore production (35). All intensities of oxidase reactions on GAA from negative to very strong were recorded. As in field isolates (see Section 3.3.2.1), growth did not occur in cultures giving very strong reactions; radial growth in other cultures was 0-38 mm. On TAA, radial growth was 18-42 mm. Positive oxidase reactions with alcoholic guaiacum depended on the presence of pigmented mycelia in cultures at the time of testing. Negative results with guaiacol were usually correlated with negative to weak reactions on GAA (Isolates B1, B2, B9, B10, B19, B26, B30) and positive results, with strong to very strong reactions on GAA (B4, B8, B12, B16, B21, B28, B29). Both negative and positive guaiacol tests were recorded among isolates which gave a moderately strong reaction on GAA (B5, B15, B18, B20, B22, B23, B25).

Obvious crusts composed of cuticular cells (Code Symbol 19), or interlocking hyphae (11), or both, were formed by only four isolates, but in only one or two cultures of each. All cultures of seventeen isolates were pigmented after 6 weeks (37); only two isolates (B10, B30) remained unpigmented (36). However, mat pigmentation was often tardy, developing after more than 3 weeks. The apparently anomalous results of negative guaiacol tests on pigmented mats (Isolates B1,B2,B15,B18,B19) can be explained by late pigment development in cultures of these isolates. The fungus induced a brown pigmentation in the medium (39) under pigmented mats only.

Table 3.5. Macroscopic and microscopic characters recorded in cultures of monobasidiospore isolations from the one basidiocarp (LB5S)

Isolate No.	Macroscopic Characters														Microscopic Characters					
	Extracellular Oxidase Reactions[a]				Obvious crusts	Nobles' Code Symbols[b]														Staghorn hyphae
	GAA		TAA	Gum Guaiacol		36	37	38	39	42	43	47	50	51	6	8	10	11	35	
B 1	++	10	+++	32	-		1[c]		4[c]		4[c]	4[c]		4[c]		4[c]	1[c]	1[c]	4[c]	2[c]
B 2	+	0	+++	35	-				4[c]		4[c]	4[c]				4[c]			4[c]	
B 4	++++	22	+++	39	+				4[c]		4[c]	4[c]				4[c]			4[c]	4[c]
B 5	+++	32	+++	42	+				4[c]		4[c]		4[c]	4[c]		4[c]			4[c]	4[c]
B 8	+++++	0	+++	29	+				4[c]		4[c]	4[c]		4[c]		4[c]			4[c]	
B 9	+	4	+++	29	-		3[c]	1[c]	3[c]	1[c]	4[c]				4[c]	4[c]			4[c]	1[c]
B10	++	7	+++	36	-		4[c]		4[c]		4[c]			4[c]	4[c]	4[c]			4[c]	
B12	+++++	0	+++	26	+			4[c]		4[c]	4[c]		4[c]			4[c]			4[c]	
B15	+++	9	+++	18	-			4[c]		4[c]	3[c]	1[c]				4[c]			4[c]	2[c]
B16	++++	15	+++	30	+			4[c]		4[c]		4[c]		4[c]		4[c]			4[c]	
B17	++	15	+++	25	-		2[c]	1[c]	3[c]	1[c]	3[c]	4[c]		3[c]	1[c]	4[c]	2[c]	1[c]	4[c]	
B18	+++	17	+++	31	-				4[c]		4[c]	4[c]		4[c]		4[c]			4[c]	3[c]
B19	++	13	+++	29	-		2[c]		4[c]		4[c]	3[c]	1[c]			4[c]	1[c]	2[c]	4[c]	3[c]
B20	+++	38	+++	42	+				4[c]		4[c]	4[c]		4[c]		4[c]			4[c]	4[c]
B21	++++	6	+++	27	+				4[c]		4[c]	4[c]		4[c]		4[c]			4[c]	2[c]
B22	+++	37	+++	40	+		1[c]		4[c]		4[c]	4[c]				4[c]		1[c]	4[c]	4[c]
B23	+++	10	+++	29	+				4[c]		4[c]		4[c]	3[c]		4[c]			4[c]	4[c]
B25	+++	17	+++	32	+				4[c]		4[c]	4[c]				4[c]			4[c]	4[c]
B26	++	14	+++	28	-		3[c]	1[c]	3[c]	1[c]	4[c]			4[c]		4[c]			4[c]	1[c]
B28	+++++	0	+++	33	+			4[c]		4[c]	2[c]	2[c]				4[c]			4[c]	
B29	+++++	0	+++	22	+			4[c]		4[c]			4[c]	4[c]		4[c]			4[c]	
B30	-	0	+++	32	-		4[c]		4[c]		4[c]			4[c]		4[c]			4[c]	

[a] Oxidase Reactions. GAA and TAA -, negative; +, very weak; ++, weak; +++, moderately strong; ++++, strong; +++++, very strong. Descriptions of these reactions are given in Appendix 6. Numbers represent radial growth (mm) in 7 days. Guaiacol: -, negative; +, positive.

[b] Code symbols (from Nobles, 1965). Macroscopic Characters: 36, white or pale mats; 37, mats yellow or brown, at least in part; 38, agar unchanged in colour; 39, agar brown at least in part; 42, plates covered in 2 weeks; 43, plates covered in 3 weeks; 47, plates not covered in 6 weeks; 50, fragrant odour; 51, earthy or musty odour. Microscopic Characters: 6, hyphae lacking clamp connections; 8, fibre hyphae; 10, cuticular cells; 11, interlocking hyphae; 35, arthrospores.

[c] Number of cultures with this character. Where number is absent no culture had this character.

Growth rates varied within and among isolates from very slow (47) to rapid (43). Pigmented cultures of some isolates (B5, B16, B23, B29) grew very slowly to moderately rapidly, but all unpigmented cultures (B9, B10, B17, B26, B30) grew rapidly.

Fibre hyphae (8) were not observed in any culture. The production of staghorn hyphae appeared to vary among isolates with pigmented cultures. Of the twenty isolates which produced pigmented cultures, thirteen were recorded to have staghorn hyphae; six of these had them in all cultures.

3.3.3 Discussion

All characters found in cultures of isolates from contexts of basidiocarps also occurred among those from host tissues and encrustations of the fungus.

The occurrence of most characters in pigmented cultures of field isolates usually varied widely. Similar wide variations in the cultural characters of field isolates have been reported for such other Hymenomycete root pathogens with wide distributions and host ranges as *Armillariella mellea* (Raabe, 1966) and *Heterobasidion annosum* (Etheridge, 1955).

Monoarthrospore isolations from the one field isolate varied in the formation and composition of crusts, in mycelial pigmentation, and in the production of staghorn hyphae. However, they all produced moderately strong oxidase reactions on both GAA and TAA and, with a single exception on GAA, grew at similar rates on the one medium. The intensities of the oxidase reactions were the same as those recorded for the parent isolate, but growth rates on both media were less (cf. Tables 3.1 & 3.3).

Monobasidiospore isolates from the one fructification varied widely in all characters, the range of variation being at least as great as that recorded for eighteen field isolates (cf. Tables 3.1 and 3.4). A similar variation has also been reported for *A. mellea*; Raabe (1966) found that cultures of 84 monospore isolates from a single basidiocarp showed as much variation as those of 84 field isolates from different origins. However, the variation among monobasidiospore isolates of *A. mellea* differs from that in *P. noxius*, as it is continuous and isolates cannot be separated into distinct cultural types (Maclean, 1950; Raabe, 1966).

3.4 CONCLUSION

A moderately strong oxidase reaction on TAA and clampless hyphae and arthrospore production in MEA cultures were characters common to all isolates of *P. noxius*. All other characters of any one field isolate of the fungus may vary widely in occurrence among replicates in a single study.

Some field isolates differed from others in intensity of the oxidase reaction on GAA, and in the intensity and extent of mat pigmentation on MEA, but there were no obvious relationships between either of these characters and the host or geographical origin, or age of an isolate. The lack of any apparent relationship between mat pigmentation and host or geographical origin of an isolate has also been observed in other pathogenic Hymenomycetes, e.g. *Armillariella mellea* (Raabe, 1966), *Heterobasidion annosum* (Etheridge, 1955) and *Phellinus igniarius, Phellinus nigricans, Phellinus populicola* and *Phellinus tremulae* (Niemelä, 1974, 1975). In the present studies, however, context isolates of *P. noxius*

were usually less pigmented than host or encrustation isolates. Comparisons between the cultural characters of isolates from basidiocarps and host tissues apparently have not been made for other Hymenomycetes.

Other pathogenic Hymenomycetes which vary widely in cultural characters, e.g. *A. mellea, H. annosum, P. populicola, P. tremulae* and *Polyporus tomentosus* also vary widely in their physiological, pathogenic or ecological behaviour (e.g. Bega & Hendrix, 1962; Hiorth, 1965; Raabe, 1967, 1972; Kuhlman, 1970; Courtois, 1974, 1978; Niemelä, 1975, 1977a; Whitney & Bohaychuk, 1977; Federov & Staichenko, 1978; Hütterman, 1978; Volger *et al.*, 1978). Further, the intensity of cultural pigmentation has been correlated with pathogenicity in *P. tomentosus* (Whitney & Bohaychuk, 1977), growth rate in *P. populicola* (Niemelä, 1975) and growth rate and enzyme production in *P. tremulae* (Hiorth, 1965; Niemelä, 1977a). Variation in the cultural characters of *P. noxius*, and particularly in the intensity of mycelial pigmentation, may reflect variation in physiology and pathogenicity within and among isolates of the fungus. Studies on physiology are described in Chapter 4 while those on pathogenicity are detailed in Chapter 5.

CHAPTER 4

VARIATION IN PHYSIOLOGY

4.1 INTRODUCTION

Phellinus noxius has been recorded on a wide range of hosts throughout most of the tropics and has been associated with a number of quite different diseases (Chapter 1). Corner (1932) described the fungus as a facultative parasite. The colonization of such a range of habitats suggests that considerable variation may exist in the physiology of the fungus.

The following experiments investigated variation in three physiological traits of *P. noxius*: (1) growth rate at various temperatures of incubation, (2) growth rate at various pH values of the medium, and (3) the production of extracellular enzymes in laboratory culture.

4.2 EXPERIMENT 1: EFFECT OF TEMPERATURE ON GROWTH RATE

4.2.1 Materials and Methods

Ten each of field, monoarthrospore, and monobasidiospore isolates were studied. Field isolates came from a variety of sources, viz. two hosts, host or basidiocarp tissues, and three geographical localities (Table 4.1). Cultures of seven of these isolates were of only the usual pigmented type, but those of the other three comprised both the pigmented type and the unpigmented variant. The latter are identified in the tables of results by the letter "U" following the isolate number. Monoarthrospore and monobasidiospore isolates were derived from the one field

collection (LB5S). The former were freshly prepared for the experiment, but the latter were taken from the culture collection (Section 2.1). Monobasidiospore isolates included representatives of each of the three cultural types (Section 3.2.2.3).

Table 4.1 Origins of field isolates

Isolate Number	Origin	Month/Year Isolated
1141C	Root of hoop pine	S.E. Queensland	3/73
1354B	Root of hoop pine	S.E. Queensland	5/74
1516	Basidiocarp on hoop pine	S.E. Queensland	6/74
2002	Basidiocarp on hoop pine	S.E. Queensland	5/75
2250	Basidiocarp on hoop pine	N. Queensland	7/75
2261	Basidiocarp on hoop pine	N. Queensland	7/75
LB5S	Basidiocarp on hoop pine	N. Queensland	3/78
LB5W	Decay, hoop pine stem	N. Queensland	3/78
LB2	Basidiocarp on rubber	Malaysia	4/56
LB3	Root of rubber	Malaysia	4/77

Growth rates were assessed in 90 mm diameter petri dishes containing 30 ml malt-extract agar (MEA: 12.5 g Difco Bacto malt-extract; 20.0 g Difco Bacto-Agar; 1000 ml distilled water; unbuffered, pH 5.7-5.8). Inoculum cultures were prepared by growing field and monobasidiospore isolates for 4 days, and monoarthrospore isolates for 7 days, from central inoculations in petri dishes of MEA. One 4 mm diameter plug cut from the advancing margin of an inoculum culture was placed mycelium down at the edge of freshly prepared dishes of MEA. Four dishes per isolate per temperature were inoculated, and incubated in the dark at 5.0±0.5°C, 10.0±0.5°C, 15.0±0.25°C, 20.0±0.25°C, 25.0±0.25°C, 27.5±0.25°C, 30.0±0.5°C, 35.0±0.5°C and 40.0±0.5°C.

At 24 hr intervals after inoculation, a line corresponding to the position of the margin of the culture was scribed on the reverse of each dish. Each line was identified with the appropriate number of days after inoculation. This procedure was usually continued until the faster growing cultures had overgrown their dishes, or for 14 days in the case of the slowest growing cultures. Where growth from an inoculum plug was tardy, scribing was continued for 14 days after first growth was observed.

Growth was measured along three radii per dish, from the scribed line corresponding to the third day of observable growth (Section 2.3). Usually, growth in 72 hr was measured for field isolates, 144 hr for monoarthrospore isolates, and 120 hr for monobasidiospore isolates. However, growth in 240 hr was measured for the slowest growing cultures. Data were converted to mm growth per 24 hr.

Where growth was not apparent after 21 days, dishes were examined at 40X magnification under a dissecting microscope. Where growth had occurred, appropriate measurements were taken. Where growth was absent, inocula were transferred to fresh dishes of MEA and incubated at 25^{o}C for 4 weeks to test viability.

Studies on field, monoarthrospore and monobasidiospore isolates were conducted separately. Field isolate LB5S was also included in each of the latter two studies for comparison with results for monospore isolates, and also for comparison of results for the same field isolate in three different studies.

4.2.2 Results

4.2.2.1 Field Isolates

Growth rates of isolates at the various temperatures of incubation

are presented in Table 4.2.

Table 4.2 Growth rate (mm/24 hr) of field isolates at various incubation temperatures

Isolate Number	Temperature of Incubation (°C)								
	5.0	10.0	15.0	20.0	25.0	27.5	30.0	35.0	40.0
1141C	0.0	0.8	3.0	5.6	9.4	10.5	11.2	2.8	0.0
1354B	0.0	0.7	5.1	8.9	11.6	7.9	7.8	3.1	0.0
1516	0.0	0.3	4.8	7.3	8.7	7.7	10.3	3.3	0.0
2002	0.0	0.6	3.4	6.4	6.8	7.4	6.9	2.6	0.0
2250	0.0	0.2	3.9	6.4	8.7	8.6	8.8	3.6	0.0
2261	0.0	0.3	3.9	4.5	6.6	6.2	6.7	3.0	0.0
LB5S	0.0	0.7	2.0	6.6	9.9	12.4	13.9	0.9	0.0
LB5W	0.0	0.9	1.4	7.4	10.7	13.8	14.7	0.3	0.0
LB2	0.0	0.3	3.5	8.7	12.0	11.3	10.6	3.0	0.0
LB3	0.0	0.4	5.5	8.2	12.0	15.1	17.3	0.3	0.0
1516U	0.0	0.3	1.2	3.6	5.2	6.6	6.3	0.0	0.0
2250U	0.0	0.2	1.6	5.3	5.2	5.9	6.0	3.5	0.0
LB3U	0.0	0.1	1.5	3.1	5.2	5.8	5.5	2.8	0.0

Values underlined are maximum growth rates for each isolate and do not differ at $p = 0.05$

Pigmented Isolates

All isolates grew over the range 10-35°C. Inocula from the 5°C treatment were still viable after 21 days incubation, but those from the 40°C treatment were not. Growth was usually observed at 24 hr after inoculation, but at 10°C, first growth was observed at 8-10 days.

The temperature promoting fastest growth varied among isolates, but optimum temperatures were all in the range 25-30°C. There was no apparent correlation between these temperatures and the origins of isolates.

The mean maximum growth rates of isolates varied from 6.7-17.3 mm/24 hr (Isolates 2261 and LB3), respectively). For these studies, and those

investigating the effect of pH on growth rate, the mean maximum growth rate is defined as the mean of the twelve determinations (three for each of four dishes in each treatment) of growth rate in the treatment which promoted fastest growth in each isolate. Both geographical origin and the type of tissue from which an isolate was derived had a significant effect on mean maximum growth rate (Table 4.3). Malaysian isolates grew faster than Queensland isolates, but there was no significant difference between north and southeastern Queensland isolates; and isolates from infected tissues of the host grew faster than those from the contexts of basidiocarps (Table 4.4).

Table 4.3 Analysis of variance on mean maximum growth rates recorded in a study on the effect of temperature on growth rates of field isolates

Source of Variation	DF	MSS	Significance of F
Geographic Origin	2	1466.524	.001
Tissue Source	1	3780.750	.001
Origin x Source	2	189.722	.004
Residual	114	32.968	

Table 4.4 Mean maximum growth rates of field isolates from different geographical and tissue sources, recorded in a study on the effect of temperature on growth rate

Source of Isolates	$\bar{x}$	s	n
Malaysia	14.6	2.7	24
N. Queensland	11.0a	3.5	48
S.E. Queensland	10.1a	1.8	48
Host	13.7	2.5	48
Basidiocarp	9.8	2.9	72

Values followed by the same letter do not differ significantly at $p = 0.05$

Unpigmented Variants

Except for the absence of growth in cultures of 1516U at 35°C, the upper and lower limits of temperature for growth and the results of tests on the viability of inocula from cultures incubated at 5°C and 40°C were similar to those of pigmented isolates (inocula of 1516U from 35°C treatment were still viable after 21 days incubation). Also, growth from inocula, where recorded, occurred within 24 hr of inoculation at all temperatures except 10°C, where it was first observed after 8-10 days.

The range of temperature optimal for growth of each variant was broader than that for its pigmented counterpart, and mean maximum growth rate was significantly slower.

4.2.2.2 Monoarthrospore Isolates

Growth rates of isolates at the various temperatures of incubation are presented in Table 4.5.

Table 4.5 Growth rate (mm/24 hr) of monoarthrospore isolates at various incubation temperatures

Isolate Number	Temperature of Incubation (°C)								
	5.0	10.0	15.0	20.0	25.0	27.5	30.0	35.0	40.0
LB5S	0.0	0.3	3.0	8.5	11.6	13.8	13.9	0.8	0.0
A1	0.0	0.3	2.7	4.0	6.2	6.1	8.0	2.3	0.0
A2	0.0	0.2	2.6	3.7	5.5	4.9	4.1	1.9	0.0
A3	0.0	0.3	2.7	3.9	4.5	4.3	4.0	2.4	0.0
A4	0.0	0.2	3.0	3.7	5.9	6.0	6.1	1.6	0.0
A5	0.0	0.3	2.7	3.7	7.1	6.1	5.8	1.9	0.0
A6	0.0	0.3	2.6	4.0	5.8	7.3	4.8	1.8	0.0
A7	0.0	0.2	2.7	3.4	5.6	6.2	5.3	1.8	0.0
A8	0.0	0.1	2.6	3.7	5.5	5.7	4.6	1.6	0.0
A9	0.0	0.3	2.8	4.1	6.0	6.8	5.8	2.0	0.0
A10	0.0	0.2	2.8	3.8	5.4	6.2	5.7	1.9	0.0

Values underlined are mean maximum growth rates for an isolate and do not differ at p=0.05

Results of the second study on isolate LB5S were similar to those of the first (Section 4.2.2.1 and Table 4.2), except that the optimum temperature was 27.5-30°C, whereas in the first it was 30°C.

All monoarthrospore isolates grew at 10-35°C, but not at 5°C or 40°C. This temperature range for growth was the same as that recorded for all field isolates (Table 4.2). However, growth from inocula incubated at 10-20°C was usually more tardy: at 10°C, growth was first observed 10 days after inoculation (cf. 8 days for LB5S); at 15°C, 48 hr; and at 20°C, 24 hr or 48 hr. At 25-35°C, growth was recorded at 24 hr. Results of tests for viability of inocula from the 5°C and 40°C treatments were as for field isolates (Section 4.2.2.1).

Temperatures optimal for growth varied considerably among isolates. Usually, they were in the temperature range 25-30°C. This range was similar to that recorded among field isolates, but broader than that (27.5-30°C) of the parent. One isolate (A3) had a very broad range of 20-30°C.

Growth rates of isolates at 10°C, 15°C, 20°C or 35°C were all remarkably similar. Mean maximum growth rates, however, varied from 4.5 to 8.0 mm/24 hr. Thus, they were considerably less than the 13.9 mm/24 hr recorded for the parent isolate. Significant differences in mean maximum growth rate occurred between isolates (Table 4.6), but only two (A1 and A3) had values which differed from those of all other isolates. The growth rate of A4 was similar to those of seven others.

Variation in mean maximum growth rate among monoarthrospore isolates was considerably less than that among pigmented field isolates. Those of the former isolates were 40-58% of LB5S and those of the latter, 48-125%.

Table 4.6 Mean maximum growth rates of monoarthrospore isolates

Isolate Number	Mean Maximum Growth Rate
LB5S	13.9a
A1	8.0 b
A6	7.3 c
A5	7.1 cd
A9	6.8 cde
A4	6.1 cdef
A10	6.2 def
A7	6.2 def
A8	5.7 ef
A2	5.5 f
A3	4.5 g

Values followed by the same letter do not differ significantly at p = 0.05

4.2.2.3 Monobasidiospore Isolates

Growth rates at the various incubation temperatures are presented in Table 4.7.

Table 4.7 Growth rate (mm/24 hr) of monobasidiospore isolates at various incubation temperatures

Isolate Number	Temperature of Incubation (°C)								
	5.0	10.0	15.0	20.0	25.0	27.5	30.0	35.0	40.0
LB5S	0.0	0.6	2.6	8.3	10.0	12.7	13.3	0.8	0.0
B1	0.0	0.5	3.1	7.3	8.9	8.0	7.8	2.3	0.0
B4	0.0	0.4	3.1	6.1	7.1	8.5	11.5	3.8	0.0
B8	0.0	0.6	2.3	5.9	7.7	8.3	9.3	2.5	0.0
B9	0.0	0.5	2.7	5.5	7.3	8.3	9.0	*	0.0
B10	0.0	0.6	3.5	5.7	7.2	8.9	9.1	3.9	0.0
B12	0.0	0.7	3.3	6.2	8.2	8.4	9.1	*	0.0
B17	0.0	0.7	3.3	5.1	7.9	7.7	7.3	2.4	0.0
B20	0.0	0.5	2.3	6.2	7.4	9.7	9.5	2.2	0.0
B28	0.0	0.2	2.8	6.4	6.3	6.5	6.4	1.9	0.0
B29	0.0	0.5	2.7	7.0	7.1	7.2	7.3	*	0.0

Values underlined are maximum growth rates for an isolate and do not differ at p = 0.05

* Bacterial contamination

Results of the third study on field isolate LB5S were similar to those of the earlier two (Section 4.2.2.2). In this study, fastest growth occurred at 27.5-30°C, the same as in the second study.

The lower and upper limits of temperature for growth were 10°C and 35°C for all monobasidiospore isolates. Growth from inocula was first observed at 48 hr for those incubated at 10°C and 15°C, and at 24 hr for those at other temperatures (20-35°C). Hence, growth from inocula at 10°C occurred earlier in monobasidiospore isolates than in field or monoarthrospore isolates (8-10 days). Results of tests for viability of inocula incubated (for 21 days) at 5°C and 40°C, were positive and negative, respectively.

Optimum temperatures for six isolates were similar to that of the context isolate from the same basidiocarp (LB5S). Another two isolates had optima of 25-27.5°C and the other two had very broad optima of 20-30°C.

Mean maximum growth rates varied from 6.5-11.5 mm/24 hr, and hence these were less than that of LB5S. Differences between some isolates were significant (Table 4.8), but only one isolate (B4), the one which grew fastest, had a growth rate different from all others. Growth rates of two isolates (B12 and B17) were each similar to those of seven others.

Table 4.8 Mean maximum growth rates of monobasidiospore isolates

Isolate Number	Mean Maximum Growth Rate
LB5S	13.3a
B4	11.5 b
B20	9.7 c
B8	9.3 cd
B10	9.1 cd
B9	9.0 cd
B1	8.9 cd
B12	9.1 cde
B17	7.9 def
B29	7.3 ef
B28	6.5 f

Values followed by the same letter do not differ significantly at p=0.05

Intermediate temperatures (20-30°C) increased the incidence of the appressed unpigmented cultural type in dimorphic isolates. At 10°C, 15°C and 35°C, all cultures were pigmented. Sectoring occurred only at 20-30°C (Table 4.9). At these temperatures five of the seven dimorphic isolates (B1, B4, B9, B10, B20) produced mats which were predominantly unpigmented. The other two isolates (B12 and B17) occasionally produced unpigmented sectors, but most cultures were entirely pigmented. Cultures of the remaining three isolates (B8, B28, B29) did not sector and remained entirely pigmented; the latter two isolates were of the appressed intensely pigmented cultural type.

Table 4.9 Number of cultures, of 4, in which sectoring was recorded in monobasidiospore isolates at various incubation temperatures

Isolate Number	Temperature of Incubation (°C)						
	10.0	15.0	20.0	25.0	27.5	30.0	35.0
B1	0	0	1	2	1	1	0
B4	0	0	1	0	0	0	0
B8	0	0	0	0	0	0	0
B9	0	0	1	2	4	1	*
B10	0	0	3	0	0	1	0
B12	0	0	0	1	0	0	*
B17	0	0	1	1	0	2	0
B20	0	0	1	3	1	0	0
B28	0	0	0	0	0	0	0
B29	0	0	0	0	0	0	*

*Bacterial contamination

Mean maximum growth rate was correlated with mycelial pigmentation in dimorphic isolates. Unpigmented mycelia grew more rapidly. The mean maximum growth rates of all dimorphic isolates with predominantly

unpigmented cultures (B1, B4, B9, B10, B20) were significantly greater than those of the two isolates (B28, B29) with intensely pigmented mycelia (Table 4.8).

Variation in mean maximum growth rate among monobasidiospore isolates (49-86% that of LB5S) was less than that among pigmented field isolates (48-125%).

4.2.3 Discussion

In these studies, pigmented field isolates from three different geographical sources (Malaysia, 5^{o}N; north Queensland, 16^{o}S and south-eastern Queensland, 27^{o}S) had the same lower (10^{o}C) and upper (35^{o}C) limits of temperature for growth, and had optimum temperatures of 25-30^{o}C. These results differ from those of Sehgal *et al.* (1966), but those workers studied only a single isolate from Sri Lanka (8^{o}N). The lower temperature limit for growth of their isolate was 18^{o}C. Unfortunately, data for 40^{o}C are missing from their results, but as a growth rate of 11.3 mm/24 hr was recorded at 36^{o}C, it seems likely that their isolate would have grown at 40^{o}C. In the present studies, all isolates failed to grow at 40^{o}C and they also apparently failed to survive 21 days at that temperature. The maximum growth (12.3 mm/24 hr) of the Sri Lankan isolate (Sehgal *et al.*,1966) was recorded at 30^{o}C, but growth rates at 25-36^{o}C were at least 80% of the maximum rate; hence, the isolate appears to have a range of optimum temperature broader than that of the isolates in the present studies.

The cardinal temperatures (minimum, optimum and maximum temperatures for growth) obtained for field isolates of *P. noxius* in the present studies appear to be consistent with what might be expected of a

hymenomycete root pathogen confined to tropical regions. A number of independent studies on *Heterobasidion annosum*, a hymenomycete root pathogen almost confined to temperate regions, have given remarkably consistent results for isolates from world-wide sources, viz. c.0°C, 22-25°C and 32°C (Cartwright & Findlay, 1934; Kamei & Hoshi, 1948; Rishbeth, 1951; Ward & Henry, 1961; Gundersen, 1962; Negruckig, 1962; Cowling & Kelman, 1964; Linnard, 1965; Gooding *et al.* 1966; Sehgal *et al.*,1966; Edmonds & Driver, 1973). On the other hand, the cardinal temperatures of *Armillariella mellea*, a root pathogen which occurs in both temperate and tropical regions, varied widely (Gibson, 1961; Rishbeth, 1968; Munnecke *et al.*,1976). Gibson (1961) recorded optimum temperatures of 22-29°C for 116 isolates of *A. mellea* from both regions, but found no correlation between optimum temperature and the climate of origin.

The results obtained for *P. noxius* appear to be consistent also with the recorded distribution in plantations of hoop pine in Queensland. In the southeast of the state, the fungus has not been observed in plantations to the west (Appendix 3). Mean daily temperatures in these plantations are lower (<19°C) than in those to the east or in north Queensland (20-25°C), where the fungus has been recorded (Bolland, 1974).

Evidence was found that the lower and upper limits of temperature for growth of *P. noxius* might be controlled rigidly within the species: the results for all field and monospore isolates were identical. Some variation existed, however, in the temperature which promoted optimum growth. Growth rates appear to be genetically determined for individual field isolates. The evidence for this includes (i) the constancy of results in three separate studies for mean maximum growth rate of the one isolate, and (ii) the narrow variation in mean maximum growth rate

recorded among monoarthrospore or monobasidiospore isolates from the one fructification, compared with that recorded among field isolates. As field isolates from Malaysia grew faster than those from Queensland, there is some evidence of environmental adaptation by *P. noxius*. The different growth rates between isolates from basidiocarps and host tissues suggest there are also inherent differences between isolates from the one geographical region. However, Etheridge (1955) and Cowling and Kelman (1964) found wide variation in growth rate among isolates of *Heterobasidion annosum* of different origins but could not correlate growth rate with origin. The findings of those authors and the lack of agreement between the results for cardinal temperatures obtained for *P. noxius* by Sehgal *et al.* (1966) and those of the present studies suggest that the temperature-growth relationships of additional isolates of *P. noxius* from world-wide sources should be studied to determine the extent of variation and correlations in the species.

The effect of temperature on the relative occurrence of the two cultural types in dimorphic basidiospore isolates similar to that in *P. noxius* has been observed elsewhere. The Bleaching type of field isolates of *Phellinus tremulae* predominates over the Staining type at optimum temperatures and has a superior growth rate; the latter type is the only isolate observed at the maximum temperature for growth of the fungus and it predominates at sub-optimum temperatures also (Niemelä,1977a).

4.3 EXPERIMENT 2: EFFECT OF pH ON GROWTH RATE

4.3.1 Materials and Methods

Fungal isolates and the procedures for assessing growth rates and the viability of inocula from treatments where growth was absent were essentially identical to those used in Experiment 1 (Section 4.2.1).

The sole modification in procedure was the assessment of radial growth rates of cultures over a 120 hr period; as in Experiment 1, growth rates for slower growing cultures were determined over a 240 hr period. The following are again emphasized: (i) monoarthrospore isolates were freshly prepared for the experiment, (ii) studies on field, monoarthrospore and monobasidiospore isolates were conducted separately and (iii) field isolate LB5S, the "parent" of monospore isolates was included also in each of the studies on monospore isolates.

Inoculum cultures were prepared by growing each isolate for 4 days from a central inoculation in petri dishes of malt-extract agar containing 15 per cent malt-extract. Growth rates were assessed in 90 mm diameter petri dishes containing 30 ml buffered malt-extract agar (12.5 g Difco Bacto malt-extract; 20.0 g Difco Bacto-Agar; 900 ml distilled water; 100 ml phosphate buffer solution). Using the series of Cummins (1928) as a guide, appropriate volumes of phosphate solutions (Appendix 8) were added to give the following target pH values: 2.0, 3.0, 4.0, 5.0, 5.5, 6.0, 7.0, 8.0 and 9.0. In each study the actual pH value of each treatment was recorded with a Beckman H2 meter after the medium was dispensed and these values are given in each table of results. Dishes were inoculated at the edge with a single fleck of aerial mycelium taken from the margin of the inoculum culture. Four replicates per isolate per pH value were inoculated and then incubated in the dark at 25±0.25^{o}C.

4.3.2 Results

4.3.2.1 Field Isolates

Growth rates of isolates at the various pH values are presented in Table 4.10.

Table 4.10 Growth rate (mm/24 hr) of field isolates at various pH values

Isolate Number	Initial pH								
	2.2	3.1	4.0	5.2	5.7	6.3	7.4	8.5	9.3
1141C	0.0	3.3	6.8	7.7	9.0	7.8	6.3	Tr	Tr
1354B	0.0	5.8	7.4	10.7	10.9	9.1	4.2	Tr	Tr
1516	0.0	2.6	6.7	8.2	8.5	8.3	3.3	Tr	Tr
2002	0.0	2.9	5.3	7.0	6.8	6.9	2.9	Tr	Tr
2250	0.0	2.6	7.3	9.1	9.6	9.0	4.5	Tr	Tr
2261	0.0	2.5	7.8	8.2	8.5	8.1	4.4	Tr	Tr
LB5S	0.0	5.3	9.6	10.6	10.8	11.0	4.9	Tr	Tr
LB5W	0.0	4.3	9.3	9.8	8.1	8.7	2.9	Tr	Tr
LB2	0.0	9.1	11.4	12.5	12.6	10.3	4.6	Tr	Tr
LB3	0.0	5.5	10.4	9.9	10.2	7.9	4.9	Tr	Tr
1516U	0.0	1.7	4.4	5.4	5.6	5.4	2.1	Tr	Tr
2250U	0.0	2.6	5.5	5.9	6.3	5.6	2.9	Tr	Tr
LB3U	0.0	3.8	4.8	5.5	4.5	4.9	3.4	Tr	Tr

Values underlined are maximum growth rates for each isolate and do not differ significantly at p = 0.05

Tr: <0.5 mm in 21 days

Pigmented Isolates

Growth occurred at all pH values from 3.1 to 9.3. Growth at pH 8.5 and 9.3 was very slow (<0.5 mm in 21 days) but observations made under 100X magnification at 7, 14 and 21 days after inoculation confirmed that growth was continuous. Although growth was not recorded at pH 2.2 after 21 days, inocula remained viable.

Optimum growth occurred over a range of pH values. This range varied between 5.2 and 6.3 for eight of the ten isolates. The lower limit of this optimum range for the other two isolates (LB5S and LB3) was at pH 4.0. Both of these isolates were from lower latitudes and

also were isolated from host, and not basidiocarp tissues. For both Malaysian isolates, the upper limit of the range of optimum pH was 5.7, values which were lower than those for all Queensland isolates. Thus, the lower and upper limits of the range of pH optimal for growth appear to differ between isolates from various sources. These findings justify further studies on additional isolates from world-wide sources.

The mean maximum growth rate (see Section 4.2.2.1 for definition) of isolates varied from 7.0-12.6 mm/24 hr. Geographical origin, but not the type of tissue from which an isolate was derived, had a significant effect on mean maximum growth rate (Table 4.11). Significant differences occurred between isolates from all geographical regions; Malaysian isolates were the fastest, and those from south-eastern Queensland the slowest growing isolates (Table 4.12).

Table 4.11 Analysis of variance on mean maximum growth rates recorded in a study on the effect of pH values on growth rates of field isolates

Source of Variation	DF	MSS	Significance of F
Geographic Origin	2	1367.724	.001
Tissue Source	1	140.083	.058
Origin x Source	2	950.021	.001
Residual	114	39.221	

Table 4.12 Mean maximum growth rates of field isolates from different geographical sources, which were recorded in a study on the effect of pH values on growth rate

Source of Isolate	$\bar{x}$	s	n
Malaysia	11.5	1.6	24
N. Queensland	9.8	1.2	48
S.E. Queensland	8.8	1.7	48

Means differ significantly at p = 0.05

All cultures of each isolate were pigmented at pH values 3.1-7.4 (at 8.5 and 9.3, growth was not discernible to the unaided eye). At pH values 3.1, 4.0 and 7.4, some cultures of six isolates (1141C, 1354B, 1516, 2250, 2261, LB3) gave rise to sectors of unpigmented mycelia which grew faster than the pigmented mycelia. The growth rate of a sector was often as great as that of cultures on media in the range of pH optima (Figure 4.1). Unpigmented sectors developed pigmentation after they overgrew the medium. Obvious sectoring was not observed at pH values in the range of pH optima (5.2-6.3). Sectoring also occurred in a check of isolate 2261 when grown on malt-extract agar buffered to pH 3.5 with 1N HCl. Hence, pigmentation appears to be influenced by the pH value rather than the buffer system.

Unpigmented Variants

The lower and upper limits of pH for growth of all three variants (3.1-9.3), and the lower and upper limits of the range of pH optimal for the growth of 1516U and 2250U (5.2-6.3) were identical to those of their pigmented counterparts. The optimum pH for LB3U was at a single value (5.2) and intermediate within the range for LB3. Inocula from treatment pH 2.2 were still viable after 21 days on the medium.

The mean maximum growth rate of each variant was significantly less than that of its pigmented counterpart.

4.3.2.2 Monoarthrospore Isolates

Growth rates of isolates at the various values of pH are presented in Table 4.13.

Figure 4.1 Fast growing unpigmented sectors arising in pigmented field isolates at suboptimum and supra-optimum pH values

Tracings of lines scribed on the underside of dishes

1-2. Isolate 1141C. Fig. 1 - Medium at pH 5.7; Fig. 2 - Medium at pH 7.4

3-4. Isolate LB3. Fig. 3 - Medium at pH 5.7; Fig. 4 - Medium at pH 3.1

All cultures at pH 5.7 were pigmented. At pH 3.1 and 7.4, fast growing sectors were unpigmented, slow growing mycelia were pigmented

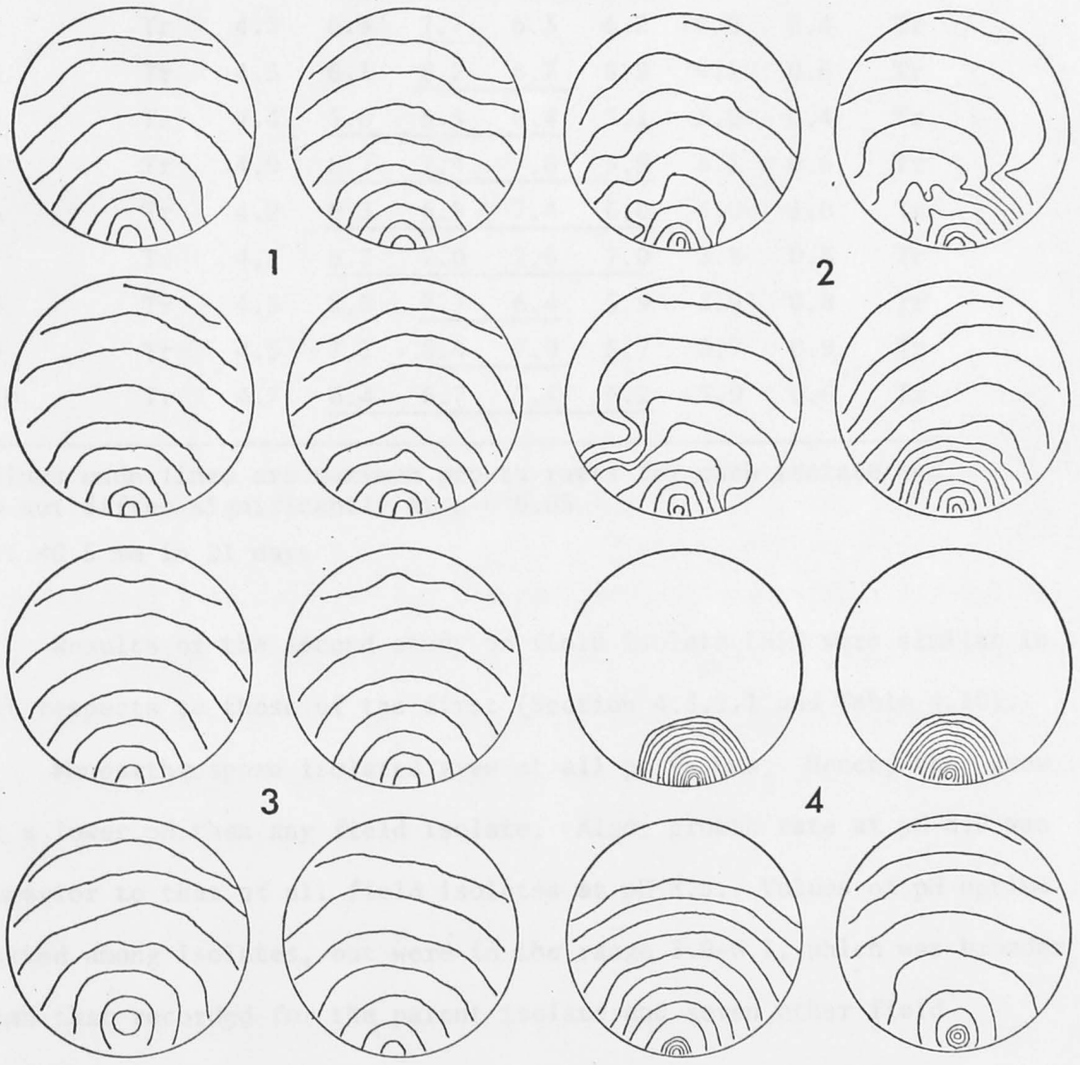

1
2
3
4

Table 4.13 Growth rate (mm/24 hr) of monoarthrospore isolates at various pH values

Isolate	Initial pH								
Number	2.2	3.1	3.9	5.2	5.8	6.2	7.5	8.9	10.0
LB5S	0.0	3.1	8.9	9.2	9.6	9.9	4.2	Tr	Tr
A1	Tr	4.7	6.2	6.3	6.7	6.1	3.9	0.1	Tr
A2	Tr	4.3	6.3	7.7	6.3	6.2	4.0	0.4	Tr
A3	Tr	4.5	6.1	8.2	6.7	5.9	4.1	0.6	Tr
A4	Tr	4.4	6.0	6.5	6.4	5.1	3.9	0.4	Tr
A5	Tr	4.9	6.7	7.4	7.8	5.9	4.1	0.6	Tr
A6	Tr	4.2	6.3	6.5	7.4	6.0	4.0	1.0	Tr
A7	Tr	4.1	6.2	7.0	7.5	7.0	3.8	0.8	Tr
A8	Tr	4.5	5.8	7.7	6.4	5.9	3.9	0.8	Tr
A9	Tr	4.5	7.2	8.4	7.9	5.7	3.7	0.9	Tr
A10	Tr	4.7	6.4	6.7	7.1	6.2	3.9	0.6	Tr

Values underlined are maximum growth rates for each isolate and do not differ significantly at $p = 0.05$

Tr: <0.5 mm in 21 days

Results of the second study on field isolate LB5S were similar in all respects to those of the first (Section 4.3.2.1 and Table 4.10).

Monoarthrospore isolates grew at all pH values. Hence, they grew at a lower pH than any field isolate. Also, growth rate at pH 8.9 was superior to that of all field isolates at pH 8.5. Values of pH optima varied among isolates, but were in the range 3.9-6.1, which was broader than that recorded for the parent isolate and seven other field isolates.

Mean maximum growth rates varied from 6.5-8.4 mm/24 hr. Differences among nine of the isolates were not statistically significant (Table 4.14). Only one isolate (A3) grew as fast as the parent isolate. The variation in mean maximum growth rate among monoarthrospore isolates (66-86% that of LB5S) was less than that among pigmented field isolates (64-115% that of LB5S).

Table 4.14 Mean maximum growth rates of monoarthrospore isolates in a study of the effect of pH on growth rate

Isolate Number	Mean Maximum Growth Rate
LB5S	9.9a
A3	8.2abc
A9	8.4 b
A5	7.8 bc
A8	7.7 bc
A2	7.7 bc
A7	7.5 bc
A6	7.4 bc
A10	7.1 bc
A1	6.7 c
A4	6.5 c

Values followed by the same letter do not differ significantly at p = 0.05

Sectoring of cultures was not observed in this study. Pigmentation of cultures of monoarthrospore isolates of LB5S is usually less well developed than that of cultures of field isolates (Section 3.2.2.2), and if "unpigmented sectors" arose in cultures of this study, they might easily have been overlooked.

4.3.2.3 Monobasidiospore Isolates

Data on growth rate at various pH values are presented in Table 4.15.

Results of the third study on isolate LB5S were similar in all respects to those of the earlier two (Section 4.3.2.2).

Monobasidiospore isolates grew at all pH values. The pH optima varied among isolates in the range 3.9-6.1.

Table 4.15 Growth rate (mm/24 hr) of monobasidiospore isolates at various pH values

Isolate	Initial pH								
Number	2.2	3.1	3.9	5.3	5.7	6.1	7.3	8.5	9.6
LB5S	0.0	4.8	6.8	9.0	9.8	8.7	4.4	Tr	Tr
B1	Tr	7.6	8.7	10.1	9.2	9.3	5.1	0.9	Tr
B4	Tr	2.9	6.8	8.8	8.1	8.3	3.6	0.7	Tr
B8	Tr	3.3	6.0	7.8	7.8	7.1	3.3	0.4	Tr
B9	Tr	5.5	6.8	6.7	7.8	7.6	5.2	1.0	Tr
B10	Tr	6.1	6.0	7.2	7.5	7.6	4.5	0.7	Tr
B12	Tr	4.6	7.0	8.7	8.9	7.8	3.5	0.6	Tr
B17	Tr	5.8	6.1	6.3	6.9	7.2	4.3	0.8	Tr
B20	Tr	2.4	6.6	8.4	7.7	7.2	3.0	0.2	Tr
B28	Tr	1.9	4.4	4.8	5.4	5.5	3.6	0.4	Tr
B29	Tr	1.8	4.2	6.6	7.2	7.1	2.5	0.1	Tr

Values underlined are maximum growth rates for each isolate and do not differ significantly at $p = 0.05$

Tr: <0.5 mm in 21 days

Mean maximum growth rates varied from 5.5-10.1 mm/24 hr. Differences were significant between some isolates (Table 4.16). Some of the dimorphic isolates with predominantly unpigmented cultures (B1, B4, B20) grew significantly faster than both isolates with intensely pigmented cultures (B28, B29). Similar differences between the growth rates of unpigmented and intensely pigmented mycelia were observed in the study on temperature-growth relations (Section 4.2.2.3). The variation in mean maximum growth rate among monobasidiospore isolates (56-103% that of LB5S) was similar to that among pigmented field isolates (64-115%).

Sectoring was observed only occasionally in cultures of dimorphic isolates, and it was recorded at all pH values from 3.1-7.3. At values outside this range, growth of cultures was insufficient for sectoring to be obvious. Observations provided no evidence that pH had an effect on

the relative occurrence of either cultural type.

Table 4.16 Mean maximum growth rates of monobasidiospore isolates in a study of the effect of pH on growth rate

Isolate Number	Mean Maximum Growth Rate
B1	10.1a
LB5S	9.8ab
B12	8.9ab
B4	8.8 b
B20	8.4 bc
B9	7.8 cd
B8	7.8 d
B10	7.6 d
B17	7.2 d
B29	7.2 d
B28	5.5 e

Values followed by the same letter do not differ significantly at p = 0.05

4.3.3 Discussion

Comparisons between the pH-growth relations of *P. noxius* and those of other Hymenomycetes are questionable as, *inter alia,* incubation temperature, medium composition and the buffer system used can influence the cardinal pH values obtained for any one isolate (Wolpert, 1924; Cummins, 1928; Child & Knapp, 1973). However, the range of pH values (3.1-9.3) over which field isolates of *P. noxius* maintained continuous growth is a little broader than that recorded for *Armillariella mellea, Heterobasidion annosum* and other wood-destroying Hymenomycetes (2.5-8.0) (Wolpert, 1924; Cummins, 1928; Etheridge, 1955; Negruckig, 1961; Ward & Henry, 1961; Gundersen, 1962; Linnard, 1965; de Azevedo & Moniz, 1974;

Courtois, 1974). Mycelial growth of *P. noxius* at pH values 8.5 and 9.3 was sparse and undetectable to the unaided eye. Such restricted growth might be undetected in liquid culture, and would doubtless be overlooked on solid media in the absence of microscopical examinations. The above reports on other Hymenomycetes contained no specific mention of such examinations where solid media were used. Hence, the actual upper pH limit for continuous growth of these fungi might well be above that reported, in which case the results for *P. noxius* would agree more closely.

The upper limits of the range of optimum pH for the two pigmented field isolates of *P. noxius* from Malaysia, and an unpigmented variant from one of the isolates, were all lower than those of field isolates and variants from Queensland. However, additionally isolates from Malaysia were from rubber, and those from Queensland were from hoop pine. Hence, differences in pH optima between isolates from the two sources may reflect adaptations by the fungus to local host or environmental factors. Such adaptations apparently occur in *Heterobasidion annosum*. Courtois (1974) and de Azevedo and Moniz (1974) reported the occurrence of two ecotypes among isolates of the fungus: those from hosts on calcareous soils had significantly higher pH optima than those from hosts on lime-deficient soils. The results obtained for *P. noxius* suggest that further studies on the pH-growth relationships of additional isolates from a variety of hosts and geographical regions may be fruitful in showing adaptation by the fungus to environmental conditions.

Most pigmented field isolates gave rise to sectors of unpigmented mycelia which appeared to be less sensitive to changes in pH than were pigmented mycelia. Such sectors arose at values of pH which were normally suboptimal or supraoptimal for the growth of an isolate; they grew faster

than pigmented mycelia and they grew at rates comparable to those recorded at the normal optimum for the isolate. These sectors developed pigmentation after they overgrew the dishes. It seems likely that such sectors would arise also under field conditions. This apparently inherent flexibility within an isolate of *P. noxius* whereby growth rate may be maintained over a wide range of pH values, would help to explain the colonization by the fungus of such a wide range of hosts and habitats within the presumably complex rainforest environment.

Monospore isolates were more tolerant than field isolates of lower and higher values of pH. They maintained continuous growth at a lower pH, and grew much more rapidly at pH 8.5-8.9. Rao (1970) has reported basidiospore germination over a pH range 2-9. This tolerance of a wide pH range, assuming it occurs also in nature, would probably confer considerable advantage on the fungus in its spread by spores as new infections may not be restricted by the pH of the substrate on which spores come to rest.

Solid media may have certain advantages over liquid media in the study of variation in the pH requirements for the growth of *P. noxius*: (i) Growth at pH extremes are more likely to be detected on solid media, and (ii) a better appreciation may be gained of within-isolate variation, e.g. the sectoring to cultural types which differed in growth rate and in response to changes in pH. However, growth appears to be more variable on a solid medium (Section 2.3, Appendix 4), and therefore differences between treatments and isolates might not be so clear. It is suggested that both solid and liquid media be used in any further studies of this nature on *P. noxius*.

4.4 EXPERIMENT 3: EXTRACELLULAR ENZYMES

4.4.1 Materials and Methods

Fungal isolates were the same as those used in the previous two experiments (Sections 4.2.1 and 4.3.1). Studies on field, monoarthrospore and monobasidiospore isolates were conducted concurrently. Monoarthrospore isolates were freshly prepared; the other isolates were taken from the culture collection.

Inoculum cultures bearing profuse aerial mycelia were prepared using the procedure described in Section 4.3.1. The procedures of Hankin and Anagnostakis (1975, 1977) were adopted to test the activity of the following ten enzymes: pectate transeliminase, pectin depolymerase, amylase, lipase, protease, phosphatase, deoxyribonuclease, ribonuclease, urease and cellulase. The essential details for preparing the various test substrates and for detecting enzyme activity are given in Appendix 7. The assays were conducted in 90 mm diameter petri dishes containing 30 ml medium. Each dish was inoculated at three places around the edge with flecks of mycelia taken from the advancing margin of an inoculum culture. Only one dish of each substrate was inoculated with an isolate. Dishes were then incubated in the dark at 25^{o}C for 4 days and following the application of appropriate reagents, each culture was carefully examined for evidence of enzyme activity.

4.4.2 Results

Only five of the ten enzymes assayed were detected among the various isolates. They were: amylase, lipase, protease, phosphatase and urease. Considerable variation in the enzymes detected occurred within and among isolates.

4.4.2.1 Field Isolates

Pigmented Isolates

Four enzymes were detected among field isolates (Table 4.17): lipase, protease, phosphatase and urease. Lipase activity was recorded in all replicates of isolates 1141C, 1354B, 1516 and LB5S, but not in 2250, LB5W and LB3; the occurrence of the enzyme varied among replicates of the remaining isolates (2002, 2261, LB2). Similarly, protease activity varied widely within and among isolates. Phosphatase was recorded in only a single replicate of one isolate (LB5S). Urease was detected in all but one isolate (LB2), and occurred in all replicates of six of the isolates. Isolates produced no more than two of the enzymes regularly (i.e. in all three replicates).

Table 4.17 Extracellular enzymes detected in cultures of field isolates

Isolate Number	Amylase	Lipase	Protease	Phosphatase	Urease
1141C	-	+	+	-	(+)
1354B	-	+	+	-	(+)
1516	-	+	(+)	-	+
2002	-	(+)	-	-	+
2250	-	-	-	-	+
2261	-	(+)	(+)	-	+
LB5S	-	+	(+)	-	+
LB5W	-	-	-	-	(+)
LB2	-	(+)	+	-	-
LB3	-	-	(+)	(+)	+
1516U	-	+	(+)	-	+
2250U	-	-	+	-	+
LB3U	-	-	+	-	(+)

\+ : Enzyme detected in all 3 replicates
(+): Variation in enzyme detected among the 3 replicates
\- : Enzyme not detected in any of the 3 replicates

The enzyme profile of an isolate could not be correlated with its origin.

Unpigmented Variants

Results of assays for each variant agreed with those of its pigmented counterpart except that protease was detected in 2250U, whereas it was absent in 2250; and phosphatase was lacking in LB3U, but was present in one replicate of LB3.

4.4.2.2 Monoarthrospore Isolates

Among the ten isolates, positive reactions were obtained for amylase, lipase, protease and urease (Table 4.18). As for the parent isolate (LB5S), lipase and urease were detected in all replicates of each monoarthrospore isolate, and protease activity was variable. Amylase was produced in cultures of only two isolates (A9, A10), whereas it was not recorded in the parent or any other field isolate. Five isolates (A1, A2, A4, A7, A8) had the one profile in which lipase, protease and urease occurred in all replicates. The other three isolates (A3, A5, A6) had another profile comprising a regular occurrence of lipase and urease, and an irregular protease activity.

Table 4.18 Extracellular enzymes detected in cultures of monoarthrospore isolates

Isolate Number	Amylase	Lipase	Protease	Phosphatase	Urease
A1	-	+	+	-	+
A2	-	+	+	-	+
A3	-	+	(+)	-	+
A4	-	+	+	-	+
A5	-	+	(+)	-	+
A6	-	+	(+)	-	+
A7	-	+	+	-	+
A8	-	+	+	-	+
A9	+	+	(+)	-	+
A10	(+)	+	-	-	+

\+ : Enzyme detected in all 3 replicates
(+) : Variation in enzyme detected among the 3 replicates
\- : Enzyme not detected in any of the 3 replicates

4.4.2.3 Monobasidiospore Isolates

All isolates produced lipase; and all but one (B29), urease (Table 4.19). Protease and phosphatase were the only other enzymes detected. The enzyme profile of most isolates appeared to be correlated with the occurrence of pigmentation in inoculum cultures (all cultures on test substrates were unpigmented). Inoculum cultures of all five isolates which produced only lipase and urease (B4, B9, B10, B17, B20) were unpigmented when inocula were taken and those of the only four isolates to produce phosphatase (B8, B12, B28, B29) were pigmented. The latter three of these isolates also produced protease. The inoculum culture of isolate B1, which formed lipase and urease regularly and protease irregularly, was unpigmented; this isolate was alone in having an enzyme profile identical to that of the context isolate (LB5S) from the parent basidiocarp.

Table 4.19 Extracullar enzymes detected in cultures of monobasidiospore isolates

Isolate Number	Amylase	Lipase	Protease	Phosphatase	Urease
B1	-	+	(+)	-	+
B4	-	+	-	-	+
B8	-	+	-	+	+
B9	-	+	-	-	+
B10	-	+	-	-	+
B12	-	+	+	+	+
B17	-	+	-	-	+
B20	-	+	-	-	+
B28	-	+	(+)	(+)	(+)
B29	-	+	+	+	-

\+ : Enzyme detected in all 3 replicates
(+) : Variation in enzyme detected among the 3 replicates
\- : Enzyme not detected in any of the 3 replicates

4.4.3 Discussion

The term "enzyme production" as used here is the synthesis of an enzyme by the fungus and the subsequent activity of the enzyme in the medium at detectable levels, thus negative results do not necessarily indicate a lack of the enzyme in an isolate. Cellulase activity was not recorded in any isolate but it is inconceivable that *P. noxius*, a wood-destroyer, does not produce the enzyme. Cellulase has been demonstrated in similar fungi (Courtois, 1974). Negative results for cellulase were obtained for all isolates of several *Agaricus* spp. (Raper & Kaye, 1978), in which the test substrate was the same as used in the present studies. Hence, the substrate may not have induced cellulase production in *P. noxius* and *Agaricus* spp. or the method may not be sufficiently sensitive to detect it. This may well apply also for some of the other enzymes not recorded in these studies.

The variation among profiles of field isolates appeared to be almost random: profiles varied widely, and there were no apparent correlations between the profile of an isolate and its origin or its cultural appearance. The variation among isolates in the production of an enzyme is apparently not unusual in the Hymenomycetes (Federov & Staichenko, 1974; Taylor, 1974; Raper & Kaye, 1978). The within-isolate variation found in *P. noxius* has also been recorded in *Phellinus tremulae* (Hiorth, 1965) and *Agaricus* spp. (Raper & Kaye, 1978).

The profiles of monobasidiospore isolates also varied widely, but evidence was found of correlations with cultural appearance. A similar correlation has been recognized in the dimorphic field isolate of *Phellinus tremulae*: the Staining cultural type produces laccase and tyrosinase and the Bleaching type, peroxidase (Hiorth, 1965).

There was greater uniformity among profiles of monoarthrospore isolates. The occurrence of amylase in two isolates appears anomalous in the light of the negative results for the enzyme in 31 other field, monoarthrospore and monobasidiospore isolates. This anomaly and the variation found among field isolates suggest that both greater replication of isolates than was used in this study and repeated studies are necessary for more definitive results.

Taylor (1974, 1977) and Hankin and Anagnostakis (1975) have proposed the use of qualitative tests for enzymes in fungal taxonomy. If such methods are to be useful, individual species must have characteristic enzyme profiles. The substrates used in the present study were the same as those used by Hankin and Anagnostakis (1975) and Raper and Kaye (1978); none of the species included by these authors had the same profile (lipase, protease, phosphatase and urease) as field isolates of *P. noxius*. Also, although the two studies had only four tests in common, only one of the 53 basidiomycete isolates investigated by Taylor (1977) had a profile similar to that of *P. noxius*. Undoubtedly, *P. noxius* would have been separated from that isolate if the studies had more tests in common. Thus, the use of enzyme profiles in taxonomy appear to have some merit.

4.5 CONCLUSION

Results of the preceding experiments confirmed that the wide variation in the cultural characters of field isolates of *P. noxius* (Chapter 3) is paralled by similar variation in a number of physiological traits in the fungus.

Certain traits (e.g. the lower and upper temperature limits, and the lower pH limit for growth) appear to be constant in all field isolates.

Others (e.g. growth rate and the pH for optimum growth) appear to be fixed for individual isolates; in these instances, differences were recorded between isolates from various sources and these suggested possible adaptations by the fungus to local host or environmental factors. Still other traits (e.g. temperatures optimal for growth and the production of extracellular enzymes) appeared to vary randomly among field isolates.

Within-isolate variation in pigmented field isolates of *P. noxius* can be partly explained by the occurrence of different cultural types which differ in certain physiological traits. Normally pigmented cultures may give rise to sectors of unpigmented mycelia which develop pigmentation when they overgrow the substrate. This sectoring was even more obvious in some monobasidiospore isolates from the one field collection of a basidiocarp (Sections 3.2.2.3, 4.2.2.3, 4.3.2.3). The studies on both field and monobasidiospore isolates revealed that unpigmented and pigmented mycelia differ in several aspects of physiological behaviour: the former (i) apparently does not produce extracellular oxidase (Sections 3.2.2.1, 3.2.2.2, 3.2.2.3, 3.3.2.2, 3.3.2.3), (ii) grows more rapidly (4.2.2.3, 4.3.2.1, 4.3.2.3), (iii) tends to predominate at temperatures optimal for growth (4.2.2.3), (iv) appears to be less sensitive to changes in pH of the substrate (4.3.2.1), and (v) has a different enzyme profile (4.4.2.3). This apparent inherent variability within isolates appears to be consistent with the wide host range and distribution of the fungus.

As mentioned previously, these experiments revealed possible physiological adaptations in *P. noxius*: isolates from rubber in Malaysia grew faster and appeared to have lower pH optima than those from hoop pine in Queensland. These results suggest that growth rate and the pH-growth relationships of additional isolates, initially from these two

sources, and then possibly from other host and geographical sources, should be examined for more conclusive evidence of adaptation. Because significant differences in growth rate were recorded between isolates from host tissues and those from the contexts of basidiocarps (Section 4.2.2.1), future investigations should study host and context isolates separately.

The possible role of the unpigmented variant in the colonization of substrates by *P. noxius* was not clarified to any degree by these experiments. The experiments revealed no apparent advantage conferred upon these variants; they were very stable and grew more slowly, and their physiological behaviour was mostly similar to that of their respective pigmented counterparts. These variants may well be mutants which arise only on the synthetic media used in laboratory culture.

CHAPTER 5

VARIATION IN PATHOGENICITY

5.1 INTRODUCTION

Phellinus noxius has been reported as a pathogen on a wide range of herbaceous plants, shrubs and trees (Chapter 1). Isolates of the fungus were found to vary widely in certain physiological traits (Chapter 4). Hence, isolates of the fungus might be expected to vary also in pathogenicity, as recorded in other hymenomycete root pathogens, e.g. *Armillariella mellea, Heterobasidion annosum* and *Polyporus tomentosus* (Raabe, 1967; Kuhlman, 1970; Whitney & Bohaychuk, 1976). Previous reports of variation in the pathogenicity of *P. noxius* were not found.

The following studies examined variation in the pathogenicity of *P. noxius* on three hosts from diverse groups of plants. Aseptic seedlings of lupin (*Lupinus angustifolius*), Monterey pine (*Pinus radiata*), and Queensland grey ironbark (*Eucalyptus drepanophylla*) were inoculated with field isolates from different host and geographical sources. Variation in the pathogenicity of monoarthrospore and monobasidiospore isolates from the one source was investigated also.

5.2 MATERIALS AND METHODS

Fungal isolates. Isolates were the same as those used in the preceding studies on physiology (Section 4.2.1). Studies on field, monoarthrospore and monobasidiospore isolates were conducted concurrently on the one host. Fresh monoarthrospore isolates were prepared for each host;

the other isolates were taken from a culture collection.

Preparation of gnotobiotic seedlings. Lupin seeds (commercial grade) were surface sterilized in 20 volume H_2O_2 for 2 hr, drained, and placed on the surface of steam sterilized moist vermiculite in aluminium trays. Additional sterile water was added to flood the vermiculite. The trays were covered with sterile aluminium foil and placed in an incubator at 25°C for 24 hr. They were then drained of excess water and returned to the incubator for a further 24-48 hr.

Pine seeds (seed lot 12176 of the CSIRO Division of Forest Research; ex Tallaganda State Forest, New South Wales) were soaked overnight in distilled water, surface sterilised in 0.1% (w/v) $AgNO_3$, washed in distilled water for 60 minutes and then rinsed in two changes of 0.5% (w/v) NaCl, followed by two changes of distilled water. All solutions and the water were sterilized by autoclaving at 121°C for 20 min prior to use. The seeds were then spread evenly over the surface of sterile moist vermiculite in aluminium trays, covered with sterile aluminium foil and incubated at 25°C for 7 days.

Ironbark seeds (seed lot 11412 of the CSIRO Division of Forest Research; ex Marlborough, Queensland) were surface sterilized using the same procedure as for pine, and placed in line on distilled water agar (1.5% agar) in 90 mm diameter petri dishes. The dishes were then placed in a plastic bag and stood on edge on the laboratory bench for 14 days, with the lines of seeds horizontal.

Inoculation. Inoculum cultures were prepared by growing isolates for 4 days from central inoculations in petri dishes containing 15 ml malt-extract agar (MEA: 12.5 g Difco Bacto malt-extract; 20.0 g Difco Bacto-Agar; 1000 ml distilled water).

Pathogenicity was assessed on seedlings in 20 cm x 2.5 cm glass test

tubes containing 15 ml unbuffered malt-extract agar (20.0 g Difco Bacto malt-extract; 15.0 g Difco Bacto-Agar; 1000 ml distilled water; pH 5.6-5.8); the agar surface was slanted 30^{o} from vertical. (The concentration of agar was found to be the minimum which held the slope and it also allowed roots to penetrate the substrate without splitting it). Tubes were fitted with aluminium caps.

One lupin or ironbark seedling, 3-4 cm long, was transferred to the substrate surface in each tube, and was allowed to establish for 2 and 21 days respectively, before inoculation. After these periods, roots had penetrated the substrate and development of laterals had commenced and shoots bore foliage as well as cotyledons. Pine seedlings were transferred to tubes after the substrate was inoculated; preliminary trials revealed that infections of pine seedlings did not occur when they were allowed to become established before inoculation. Lupin and ironbark seedlings were inoculated at the root collar with a 4 mm diameter plug cut from the advancing margin of inoculum cultures. Pine seedlings were placed on growing cultures. A control treatment (uninoculated host seedlings) was included in each of the studies with inocula comprising plugs of agar cut from a sterile dish of MEA. Ten seedlings of each host were inoculated with each isolate. Separate studies were conducted on the three hosts.

Incubation. Tubes were randomly located on stands in an Email LBH controlled environment cabinet. Incubation was at 25^{o}C. Continuous light was provided by fluorescent tubes emitting a 4 00 -7 00 nm wavelength spectrum at an intensity of 350-400 $\mu E\ m^{-2}sec^{-1}$. Direct sunlight readings at the time were 2,000-2,500 $\mu E\ m^{-2}sec^{-1}$. Light intensity readings were made with a Li-Cor LI-170 Quantum radiometer/photometer fitted with a quantum sensor. Humidity was not controlled but it was about 100% within

tubes as evidenced by the continuous presence of free water on the inside walls.

Observations. Seedling symptomatology and changes in the appearance of fungal cultures were recorded daily for the duration of each study. Studies were terminated when seedlings in control treatments began to show symptoms of stress, presumably induced by the exhaustion of nutrients or moisture in the substrate. Hence, the study with lupin was terminated 21 days after inoculation and those with pine and ironbark, 42 days after inoculation.

Re-isolations of the fungus were attempted as mortalities occurred, and also from inoculated, but apparently healthy seedlings, at the completion of a study. Stems of seedlings were freed of superficial mycelia and a section, 1 cm in length, was cut from the base of the stem. Sections were surface sterilized in 2.5% NaOCl for 30 sec and then plated on fresh MEA. Transverse sections were cut also from lupins for observations on penetration of tissues by mycelia under the scanning electron microscope.

Assessments of pathogenicity. The pathogenicity of isolates was assessed quantitatively as (i) per cent mortality and (ii) virulence. The virulence of an isolate was calculated by the mean of "virulence indices" for individual seedlings. The method for determinations of virulence indices was given in Section 2.6.3.

5.3 RESULTS

5.3.1 Symptomatology

Lupin. The first observable symptoms were water-soaked necrotic lesions around the root collar. The lesions expanded and soon girdled the seedling. Foliar symptoms then became apparent, and proceeded from

wilting, to partial loss of older foliage and withering of remaining foliage, and finally to death. The lesions continued to extend up the stem, and reached the crown some time after the death of the foliage. Lesions on stems and exposed roots bore profuse superficial mycelia, almost to the advancing margin; those on submerged roots carried only sparse superficial mycelia. Seedlings were recorded as dead when they were entirely necrotic above the root collar.

Pine. First symptoms were necrotic lesions around the root collar. Foliar symptoms appeared only after stem necrosis had extended to the cotyledons. Necrosis of the cotyledons proceeded from their proximal to their distal parts. Primary needles then began to turn brown from their bases, often before cotyledons were completely brown. Profuse superficial mycelia occurred on lesions around the root collar, but only occasionally on lesions on stems and foliage. Seedlings were recorded as dead when they were entirely necrotic above the root collar.

Ironbark. Observations on first symptoms were confined to the foliage because seedlings were small (the height of seedlings from the root collar was mostly 1-2 cm) and the profuse aerial mycelia on the substrate completely enveloped the lower stem. Foliar symptoms progressed from the apices of individual leaves to their bases, and from the oldest foliage to the youngest. Individual leaves firstly turned chlorotic, and then withered and died. Stems then began to pale and turn brown.

Seedlings sometimes dropped their dead foliage and produced new healthy shoots. In such instances, stems did not pale in colour and seedlings remained healthy. Hence, ironbark was not recorded as showing first symptoms until stems began to pale. Seedlings were recorded as dead when leaves and stems were entirely necrotic. As with pine, obvious superficial mycelia occurred only occasionally on stems and foliage.

5.3.2 Recovery of the Pathogen

Phellinus noxius was recovered from all dead seedlings. It was not isolated from healthy seedlings in treatments where inocula comprised unpigmented variants of field isolates, nor from any ironbark seedling which defoliated and produced new healthy shoots. As *P. noxius* was the only organism isolated from diseased seedlings and uninoculated control seedlings remained healthy for the duration of each study, the fungus was considered to be the causal agent of the disease encountered in these studies.

5.3.3 Pathogenicity of Isolates on the Three Hosts

5.3.3.1 Lupin

Per cent mortality and virulence indices for the various isolates are presented in Tables 5.1 and 5.2, respectively.

Table 5.1 Per cent mortality of lupin caused by various field and monospore isolates

Field Isolates		Monoarthrospore Isolates		Monobasidiospore Isolates	
1141C	100	LB5S	70	LB5S	70
1354B	100	A9	20	B8	50
2002	100	A1	10	B4	40
LB2	100	A4	10	B28	30
LB3	100	A5	10	B20	20
LB5W	100	A6	10	B10	10
2261	90	A10	10	B1	0
LB5S	70	A2	0	B9	0
2250	20	A3	0	B12	0
1516	0	A7	0	B17	0
1516U	0	A8	0	B29	0
2250U	0				
LB3U	0				
CONTROL	0				

Table 5.2 Virulence indices for various field and monospore isolates on lupin

Field Isolates		Monoarthrospore Isolates		Monobasidiospore Isolates	
1141C	4.0a	LB5S	3.4a	LB5S	3.4a
1354B	4.0a	A1	2.2 b	B8	3.0ab
2002	4.0a	A10	2.0 bc	B4	2.8ab
LB2	4.0a	A9	1.7 bcde	B28	2.6abc
LB3	4.0a	A4	1.5 bcde	B20	2.3abcd
LB5W	4.0a	A6	1.5 bcde	B10	2.2 bcd
2261	3.8ab	A2	1.2 cde	B9	2.0 c
LB5S	3.4 b	A3	1.1 cde	B12	2.0 c
2250	1.7 c	A5	1.0 cde	B17	2.0 c
1516	1.1 c	A7	0.5 de	B1	1.8 cd
1516U	0.0 d	A8	0.4 e	B29	1.3 d
2250U	0.0 d				
LB3U	0.0 d				
CONTROL	0.0				

Values in the one column followed by the same letter do not differ significantly at p = 0.05

Wide variation in pathogenicity was recorded among normal field isolates: mortality varied from 0% to 100%, and virulence indices, between 1.1 and 4.0. Lupin appeared to be very susceptible to most isolates: six isolates caused 100% mortality, and indices of the seven most virulent isolates were similar. The source of an isolate had a significant effect on virulence (Table 5.3): isolates from Malaysia were more virulent than those from Queensland, and those from tissues of the host were more virulent than those from the contexts of basidiocarps (Table 5.4). None of the unpigmented variants was pathogenic.

Table 5.3 Analysis of variance on virulence indices of pigmented field isolates on lupin

Source of Variation	DF	MSS	Significance of F
Geographic Origin	2	3.526	.045
Tissue Source	1	22.003	.001
Origin x Source	2	3.515	.045
Residual	94	1.105	

Table 5.4 Virulence indices on lupin of pigmented field isolates from different tissue and geographical sources

Sources of Isolates	$\bar{X}$	s	n
Malaysia	4.0	0.0	20
S.E. Queensland	3.3a	1.3	40
N. Queensland	3.2a	1.3	40
Host	4.0	0.0	40
Basidiocarp	3.0	1.4	60

Values followed by the same letter do not differ significantly at p = 0.05

Seventy per cent mortality and a virulence index of 3.4 were recorded for the parent (LB5S) of monospore isolates. Monoarthrospore isolates caused a lower mortality (0-20%) and were significantly less virulent (indices of 0.4-2.2) than the parent. Monobasidiospore isolates caused a lower mortality (0-50%) than the parent, and virulence indices (1.3-3.0) were also lower; however, the indices of the four most virulent isolates were statistically similar to that of the parent.

5.3.3.2 Pine

As for lupin, results for mortality (Table 5.5) and virulence (Table 5.6) varied widely among normal field isolates. Also, the source of an isolate again had a significant effect on virulence (Table 5.7): isolates from Malaysia and southeastern Queensland were more virulent than those from north Queensland, and those from host tissues were more virulent than those from the contexts of basidiocarps (Table 5.8). Unpigmented variants again appeared to be non-pathogenic.

Isolate LB5S was the only field isolate which failed to induce disease on pine seedlings. Monospore progeny of this isolate also showed a similar lack of pathogenic capability on this host.

Table 5.5 Per cent mortality of Monterey pine caused by various field and monospore isolates

Field Isolates		Monoarthrospore Isolates		Monobasidiospore Isolates	
1141C	90	LB5S	0	LB5S	0
LB3	80	A1	0	B1	0
2002	50	A2	0	B4	0
LB2	50	A3	0	B8	0
1354B	30	A4	0	B9	0
2261	20	A5	0	B10	0
2250	10	A6	0	B12	0
LB5W	10	A7	0	B17	0
1516	10	A8	0	B20	0
LB5S	0	A9	0	B28	0
1516U	0	A10	0	B29	0
2250U	0				
LB3U	0				
CONTROL	0				

Table 5.6 Virulence indices for various field and monospore isolates on Monterey pine

Field Isolates		Monoarthrospore Isolates		Monobasidiospore Isolates	
1141C	5.3a	LB5S	0.0a	LB5S	0.0a
LB3	4.6ab	A1	0.0a	B1	0.0a
2002	3.7abc	A2	0.0a	B4	0.0a
1354B	3.0 bcd	A3	0.0a	B8	0.0a
LB2	3.0 bcd	A4	0.0a	B9	0.0a
2250	2.1 cd	A5	0.0a	B10	0.0a
2261	1.2 de	A6	0.0a	B12	0.0a
LB5W	0.5 e	A7	0.0a	B17	0.0a
1516	0.3 e	A8	0.0a	B20	0.0a
LB5S	0.0 e	A9	0.0a	B28	0.0a
1516U	0.0 e	A10	0.0a	B29	0.0a
2250U	0.0 e				
LB3U	0.0 e				
CONTROL	0.0				

Values in the one column followed by the same letter do not differ significantly at p = 0.05

Table 5.7 Analysis of variance on virulence indices of pigmented field isolates on Monterey pine

Source of Variation	DF	MSS	Significance of F
Geographic Origin	2	53.485	.001
Tissue Source	1	31.211	.004
Origin x Source	2	18.594	.007
Residual	94	3.487	

Table 5.8 Virulence indices on Monterey pine of pigmented field isolates from different geographical and tissue sources

Source of Isolate	$\bar{X}$	s	n
Malaysia	3.8a	2.0	20
S.E. Queensland	3.1a	2.3	40
N. Queensland	1.0	1.8	40
Host	3.4	2.4	40
Basidiocarp	1.7	2.1	60

Values followed by the same letter do not differ significantly at p = 0.05

5.3.3.3 Ironbark

Pigmented field isolates again varied widely in mortality induced (Table 5.9) and in virulence (Table 5.10) and source also again had a significant effect on the virulence of isolates (Table 5.11). However, differences in virulence between isolates from the three geographic sources were more clear than in the previous studies: isolates from Malaysia were significantly more virulent than those from southeastern Queensland, and those from north Queensland were significantly the least virulent (Table 5.12). Unpigmented variants again failed to show apparent disease.

Table 5.9 Per cent mortality of ironbark caused by various field and monospore isolates

Field Isolates		Monoarthrospore Isolates		Monobasidiospore Isolates	
LB3	60	LB5S	20	LB5S	20
1141C	50	A6	20	B4	20
1354B	50	A7	20	B8	10
LB2	50	A8	20	B10	10
2002	20	A2	10	B20	10
LB5W	20	A9	10	B1	0
LB5S	20	A1	0	B9	0
2261	0	A3	0	B12	0
2250	0	A4	0	B17	0
1516	0	A5	0	B28	0
1516U	0	A10	0	B29	0
2250U	0				
LB3U	0				
CONTROL	0				

Table 5.10 Virulence indices for various field and monospore isolates on ironbark

Field Isolates		Monoarthrospore Isolates		Monobasidiospore Isolates	
LB3	4.9a	A8	2.7a	B29	2.4a
1141C	4.5a	A2	1.5a	B20	2.3a
LB2	4.0ab	A7	2.0ab	B17	1.9a
1354B	3.5abc	A6	1.5ab	B12	1.9a
LB5W	2.4abcd	LB5S	1.4ab	B4	2.0ab
2002	2.4abcd	A9	1.4ab	LB5S	1.4ab
2261	2.2 bcd	A10	1.2ab	B10	1.4ab
LB5S	1.4 cde	A5	1.0ab	B28	1.4ab
2250	1.1 de	A1	0.9ab	B8	1.2ab
1516	0.6 e	A3	0.7ab	B9	1.2ab
1516U	0.0 f	A4	0.3 b	B1	0.4 b
2250U	0.0 f				
LB3U	0.0 f				
CONTROL	0.0				

Values in the one column followed by the same letter do not differ significantly at p = 0.05

Table 5.11 Analysis of variance on virulence indices of pigmented field isolates on ironbark

Source of Variation	DF	MSS	Significance of F
Geographic Origin	2	35.439	.001
Tissue Source	1	58.403	.001
Origin x source	2	7.153	.154
Residual	94	3.774	

Table 5.12 Virulence indices on ironbark of pigmented field isolates from different geographical and tissue sources

Source of Isolates	$\bar{X}$	s	n
Malaysia	4.5	2.5	20
S.E. Queensland	2.8	2.4	40
N. Queensland	1.8	1.5	40
Host	3.8	2.4	40
Basidiocarp	2.0	1.9	60

All values were significantly different at $p = 0.05$

Percent mortality recorded among monospore isolates varied from 0% to 20% and thus were comparable to that recorded for the parent (20%). Some significant differences in virulence occurred among monoarthrospore and monobasidiospore isolates, but in both instances, all isolates had similar indices to that of the parent.

5.3.4 Correlation between Pathogenicity and Mycelial Pigmentation

Where isolates were shown to be pathogenic, pathogenicity was associated with the occurrence of pigmented mycelia. Disease was recorded only in tubes containing pigmented cultures and was not observed in those where inocula of the three unpigmented variants were used, or where individual cultures of an isolate remained unpigmented (Figures 5.1 and 5.2).

Figure 5.1 Photographs illustrating the relationship between the occurrence of mycelial pigmentation and disease of lupin

1. Isolate LB3. All replicate cultures developed mycelial pigmentation early, and all seedlings died.
2. Isolate LB3U. All replicate cultures remained unpigmented, and all seedlings remained healthy.
3. Isolate 2250. Replicate cultures varied in the occurrence of mycelial pigmentation. Three of the cultures depicted in the photograph are pigmented, and seedlings are dead or dying; and the other three cultures are unpigmented, and seedlings are healthy. One of the latter cultures later developed pigmentation, and the seedling subsequently became unhealthy.

Photographs taken 14 days after inoculation


1

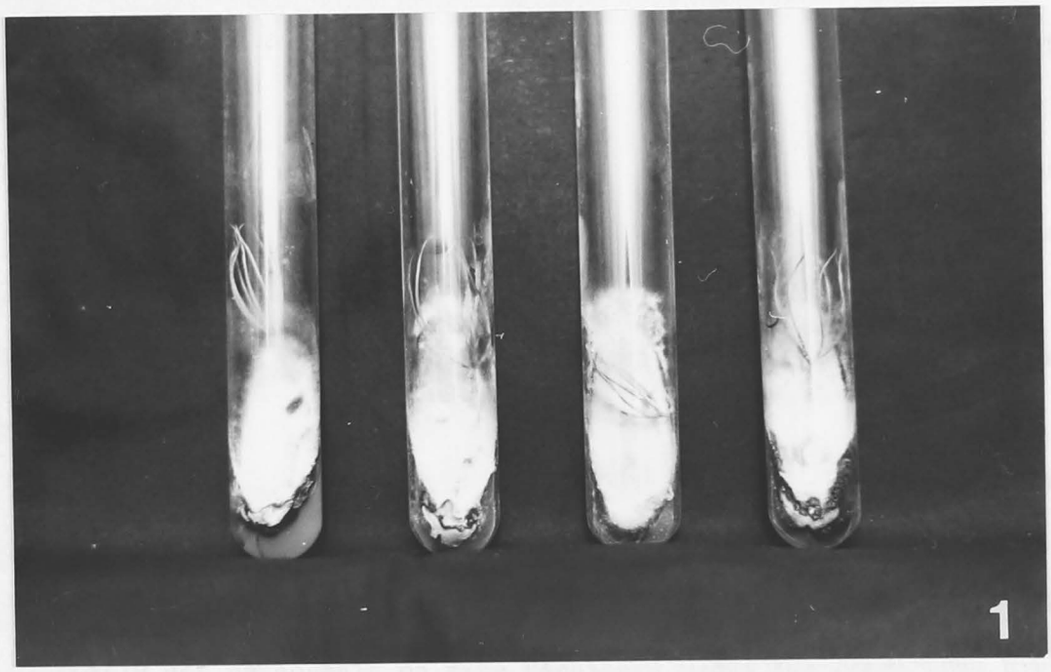

2


3

Figure 5.2 Photographs illustrating the relationship between the occurrence of mycelial pigmentation and disease of Monterey pine

1. Isolate LB3. All replicate cultures developed mycelial pigmentation early.
2. Isolate LB3U. All replicate cultures remained unpigmented, and all seedlings remained healthy.

Photographs were taken 42 days after inoculation.


1


2

Highly pathogenic isolates (e.g. 1141C and LB3) regularly produced pigmentation in young cultures, while other less pathogenic isolates (e.g. 1516, 2250 and all monoarthrospore isolates) comprised replicate cultures in which the development of pigmentation was tardy. From observations on these latter isolates, it was apparent that seedlings failed to show symptoms of disease until a culture developed pigmentation (Figure 5.1.3). Examination of lupin sections under the scanning electron microscope revealed that penetration of host tissues may not occur until after mycelia become pigmented (Figure 5.3).

The pathogenicity of an isolate is not, however, solely dependent on the occurrence of mycelial pigmentation. Many replicate cultures of isolate LB5S and its monospore progeny were pigmented but the isolate is apparently not pathogenic on pine seedlings under the conditions prevailing in these studies; the fungus was not recovered from inoculated seedlings, and necrotic tissues were not observed in dissections of such seedlings.

5.4 DISCUSSION

These studies showed that isolates from the contexts of basidiocarps of *P. noxius* are significantly less virulent than those from the tissues of the host. This correlation between virulence and these two sources of isolates has not previously been reported for hymenomycete root pathogens. Where differences have been reported in the pathogenicity of isolates from the same host species and locality, the precise origin of isolates usually has not been clearly stated. The present studies indicate that such information is important.

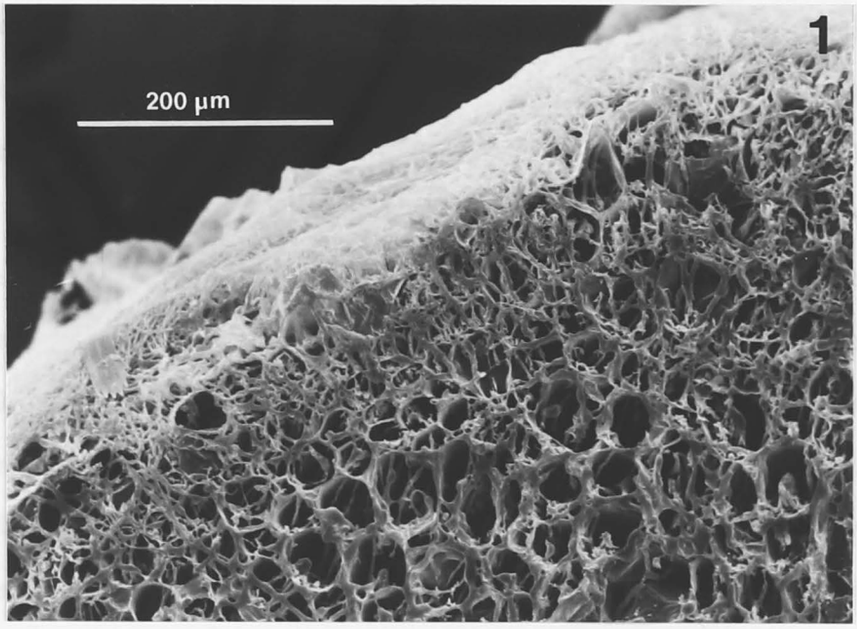
Isolates of *P. noxius* from north Queensland were consistently among the less virulent. These results may be partly due to the numerical

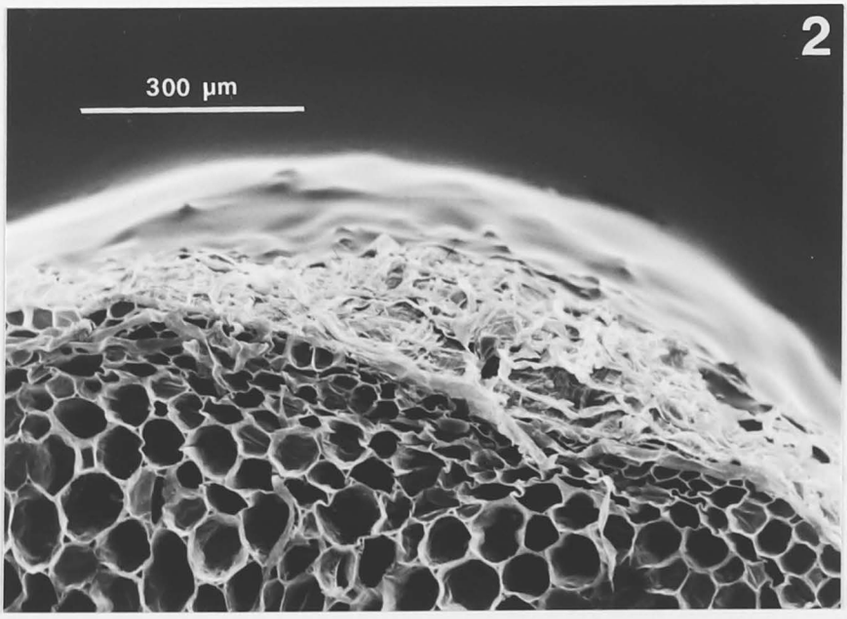
Figure 5.3 Electronmicrographs of sections removed from the root collars of lupin inoculated with isolate 2250

1. Seedling removed from a pigmented culture. Hyphae have penetrated the host

2. Seedling removed from an unpigmented culture. Hyphae are superficial and no penetration of the host has occurred

Sections of both seedlings were removed 14 days after inoculation


1
200 µm


2
300 µm

imbalance of isolates from host and basidiocarp tissues which comprised the sample. The samples from both Malaysia and southeastern Queensland had an equal number of isolates from host tissues and from basidiocarps, but three of the four isolates from north Queensland were of the less pathogenic context basidiocarp isolates. However, geographical origin appears to have a significant effect on the virulence of isolates, e.g. isolates from Malaysia were significantly more virulent than those from southeastern Queensland, on both lupin and ironbark. A similar variation in virulence is reported among isolates of *Heterobasidion annosum* from different localities (Kuhlman, 1970); isolates of the fungus from Australia, New Zealand and Norway were less virulent on seedlings of *Pinus taeda* L. than were those from the United States, India and England.

Malaysian isolates were from rubber, while those from Queensland were from hoop pine. The differences in virulence of isolates from these two sources may reflect local adaptations by the fungus; if so, it is not clear whether the adaptations are due to host or other environmental influences. Studies on other hymenomycete root pathogens (e.g. *Armillariella mellea, H. annosum* and *Polyporus tomentosus*) suggest that the original host may not be a significant factor in differences between the pathogenicity of isolates (Raabe, 1967; Kuhlman, 1970; Whitney & Bohaychuk, 1977).

Isolates of *P. noxius* derived from the same tissue, host, and geographical source differed in virulence. Isolates 1141C and 1354B were both isolated from infected roots of hoop pine in southeastern Queensland, but the virulence of the former was greater than that of the latter on both pine and ironbark and was significantly greater on pine. The virulence of context isolates from the one locality also varied. Of the two isolates from basidiocarps in southeastern Queensland, one (2002)

was significantly more virulent than the other (1516) on all three hosts; also, significant differences on both lupin and pine were recorded among the three isolates (2250, 2261, LB5S) from north Queensland. The studies of Whitney and Bohaychuk (1976, 1977) on *P. tomentosus* suggest that investigations into the occurrence of taxonomic varieties among basidiocarps of *P. noxius* may be worthwhile. Those authors found that isolates derived from two varieties of *P. tomentosus*, which could not be separated on cultural features, differed significantly in virulence on a number of test hosts; the distinction between the two varieties is based solely on the shape of hymenial setae.

The pathogenicity of *P. tomentosus* is apparently altered when taken from its natural woody substrate and cultured on standard media (Whitney & Bohaychuk, 1977). There was no evidence of such an alteration in *P. noxius*. The two most virulent isolates (LB3 and 1141C) had been cultured for 1 and 5 years, and the least virulent isolates had been cultured for between 2 months and 4 years. Hence, the pathogenicity of an isolate of *P. noxius* may be retained over long periods on synthetic media.

Correlations between the cultural appearance of isolates of hymenomycete root pathogens, and pathogenicity, have rarely been investigated. Raabe (1967, 1972) sought such relationships in *A. mellea*, but found none. Whitney and Bohaychuk (1977) however, found a positive correlation between the intensity of mycelial pigmentation of isolates of *P. tomentosus* and pathogenicity, and these relationships were independent of, *inter alia*, culture age and sporophore morphology. In the present studies, a similar relationship was apparent in *P. noxius*; good evidence was found that mycelia of field and monospore isolates were non-pathogenic while they remained unpigmented, but disease symptoms became manifest after they

developed pigmentation.

It is not clear from these studies if the comparatively low virulence of monospore isolates is inherited from its weakly pathogenic parent or reflects the low virulence of such isolates generally. The latter case appears to apply to *A. mellea*. Raabe (1972) found that monobasidiospore isolates were less virulent than the parent isolate; the parent isolate was the most virulent of ten field isolates comprising an earlier study (Raabe, 1967). A comparison of the results of the two studies reveals that the virulence of monospore isolates was generally lower than that of field isolates. Additional studies on monospore isolates of more virulent field isolates are required to clarify the situation in *P. noxius*.

As in the studies on the physiology of the fungus (Section 4.5), the role of the unpigmented variant in the colonization of substrates by *P. noxius* was not clarified in these studies, and the suggestion made previously that these variants may be laboratory mutants appears plausible.

The inoculation technique used in these studies proved very satisfactory. It offers many advantages over conventional glasshouse and field studies: (i) large numbers of plants can be processed simultaneously, allowing for increased replication; (ii) the percentage of successful infections by very pathogenic isolates can be high, and so significant differences in virulence between isolates are more likely; (iii) interference by contaminating organisms is minimal (<1% in these studies); (iv) many of the host and environmental factors which contribute to the resistance of plants in the glasshouse and field are eliminated; and (v) a better appreciation can be gained of any variation which may be due to differences in pathogenicity among cultural types. The technique is recommended for investigating variation in the pathogenicity of isolates of other *Phellinus* spp., and possibly other hymenomycete root pathogens.

CHAPTER 6

NUCLEAR LIFE HISTORY

6.1 GENERAL INTRODUCTION

In preceding experiments, isolates of *P. noxius* were found to vary widely in such characters as cultural appearance, physiology and pathogenicity. Cultures of the fungus appeared to be in a state of flux; Unpigmented sectors frequently occurred in the usual pigmented culture of field isolates, and spontaneous reversible changes between unpigmented and pigmented mycelia were recorded in appressed cultural types of monobasidiospore isolates (Chapter 3). Differences were found between unpigmented and pigmented mycelia in various physiological traits (Section 4.5) and in pathogenicity (Section 5.4). These observations suggested that *P. noxius* might possess considerable flexibility when confronted with changing environments. In addition, stable unpigmented variants were isolated from the usual pigmented culture of some field isolates; these variants were, *inter alia*, slower growing than the normal isolate and were non-pathogenic. Since the variants appeared to have no apparent advantage in physiology or pathogenicity over the usual pigmented isolates, it was suggested they might be laboratory mutants (Sections 4.5 and 5.4).

Apart from the usual sexual cycle, it is accepted that mutation, heterokaryosis, parasexuality, physiological adaptation and cytoplasmic determinants provide variation in fungi (Webster, 1974). Heterokaryosis is the subject of a separate study (Chapter 7) in this series of experiments on variation in *P. noxius*. The experiments described below examined

the nuclear condition and ploidy levels in spores and mycelia and nuclear division in the sexual and somatic phases of the fungus. Isolates studied came from host and basidiocarp tissues and from single sexual and asexual spores. A number of nuclear mechanisms which explain part of the variability exhibited by *P. noxius* were recognized.

6.2 EXPERIMENT 1: CYTOLOGICAL STUDIES ON HYPHAE IN CULTURES DERIVED FROM BASIDIOCARP AND HOST TISSUES, AND IN COLONIES ARISING FROM SINGLE ARTHROSPORES AND BASIDIOSPORES

6.2.1 Introduction

In preliminary experiments on various procedures for staining nuclei in somatic hyphae of *P. noxius*, it was noted that hyphal cells were multinucleate, whereas arthrospores had a variable, but predominantly uninucleate condition. These observations suggested that as well as studying the nuclear condition of mycelia of tissue isolates, the development of the multinucleate condition in hyphae derived from spores with single or low numbers of nuclei should be followed. The experiment comprised three parts: (1) an examination of the nuclear condition of various cells in mycelia of cultures from basidiocarp and host tissues; (2) a study of the cytology of spore germination and the subsequent development of colonies from single arthrospores; and (3) a study similar to (2), but on germination of basidiospores and the subsequent development of colonies from them.

6.2.2 Materials and Methods

Most of the fungal material came from a north Queensland collection of a hoop pine stem section bearing a basidiocarp. This basidiocarp provided a context isolate (LB5S), and basidiospores from which monosporous

isolates and spore suspensions were prepared; the context isolate was used to prepare monoarthrospore isolates and arthrospore suspensions. Additional fungal material came from the context of a basidiocarp on another hoop pine stem in north Queensland (2250) and from the root of an infected rubber tree in Malaysia; the latter comprised a pigmented isolate (LB3) and an unpigmented variant (LB3U).

Inoculum cultures of context, host tissue and monosporous isolates were prepared by growing each isolate for 4 days from a central inoculation in 90 mm petri dishes containing 15 ml malt-extract agar (MEA: 12.5 g Difco Bacto malt-extract; 20.0 g Difco Bacto-Agar; 1000 ml distilled water). For mass spore inoculations, fresh undiluted suspensions of arthrospores and basidiospores were prepared in the manner described earlier (Section 2.1).

Discs, 70 mm diam, were cut from a cellophane membrane, boiled in distilled water for 30 minutes, washed in three changes of cold distilled water, interleaved with moist filter paper, and sterilized by autoclaving at 121°C. One disc was placed on the surface of MEA in 90 mm diam petri dishes containing 25 ml medium.

Inocula for context, host tissue and monosporous isolates comprised 4 mm diam plugs cut from the advancing margins of inoculum cultures. One plug was placed near the edge of the cellophane disc in each dish. Dishes were then incubated for 96 hr at 25°C in the dark.

For studies on colonies developing from spores, 0.1 ml spore suspension was placed at the centre of each cellophane disc; because free moisture was present on the cellophane surface, the suspension spread out from the point of inoculation thus diluting the suspension. Dishes were incubated at 25°C in the dark for 0, 3, 6, 12, 18, 24 or 48 hr.

At the end of each incubation period, the appropriate dishes were

removed from the incubator and a single strip of cellophane, 40 mm x 10 mm, bearing fungal material, was cut from each disc. Where plug inoculum was used, the strip was cut along the radius of the culture and included the advancing margin. Where mass spore inoculum was used, dishes incubated for up to 24 hr were examined under 10X magnification and the strip was removed from the most dense concentration of fungal material. In dishes incubated for 48 hr, fungal growth was obvious to the unaided eye; the strip was removed from the margin of the population of colonies where, in later microscopical observations, the lower occurrence of hyphal intermingling between colonies allowed the ready isolation of hyphal tips in the advancing margins of individual colonies.

Fixing and nuclear staining were undertaken using the method described by Chang (1977). Cellophane strips with attached fungal material were dropped into 1% (w/v) mercuric chloride in absolute methanol pre-chilled to c.-60^{o}C in dry ice, and left in the solution maintained at that temperature for 3-4 days. They were then returned to room temperature for 30 minutes, washed three times (for 4, 4 and 10 hr) in absolute methanol, dipped in 1% (w/v) celloidin dissolved in a 1:1 mixture of absolute ethanol and diethylether, and passed through a graded ethanol series to distilled water (3 min per change). Nuclei were stained by immersion for 2-4 min in a fresh solution (2-3 hr old) of Mayer's haemalum containing 0.1% haematoxylin, 5.0% potassium aluminium phosphate and 0.02% sodium iodate (all w/v), in distilled water. The strips were then washed in three changes (each of 1 min) of distilled water and mounted in 50% glycerol on glass microscope slides. Material was usually examined within 2-4 days of mounting, but when coverslip edges were sealed with cosmetic nail varnish and the slides were stored in the dark, preparations remained in good condition for at least 6 months.

Slides were examined with a Wild M20 microscope using bright field optics. Micromorphological features of basidiocarp, host tissue, and monosporous isolates were recorded, particular attention being paid to hyphal diameters, septation and anastomoses in various parts of each culture; measurements of diameters and cell lengths were made with the microscope fitted with an eyepiece scale calibrated with a stage micrometer. Also recorded were the number of nuclei in apical and subapical (penultimate) cells in the advancing margins of cultures, cells of the older mycelia sampled with the cellophane strip and arthrospores. When necessary, better definition of nuclei was achieved by using a red filter on the microscope.

Similar records of micromorphology and nuclear condition were taken of spore germination and the development of colonies from single arthrospores and basidiospores in mass spore inoculations. These were taken at the end of each of the various incubation periods.

The number of cells, etc. sampled for counting varied; these data accompany tables in the text. Selected material was photographed with a Carl Zeiss photomicroscope using bright field optics and a green filter; either Kodak Panatomic X or Agfa Copex Pan rapid film was used.

6.2.3 Results

6.2.3.1 Cultures Derived from Basidiocarp and Host Tissues

Micromorphology. Crustose areas had not been produced by any culture at the time material was taken for examination. However, mycelia of all cultures, except those of the unpigmented variant, had developed pigmentation behind the advancing margin; some of these pigmented hyphae were included in the material. All pigmented isolates had similar mycelial

morphology, however, mycelia of the unpigmented variant differed in some respects. These differences are specifically mentioned in the text.

All cultures had two types of hyphae (Fig. 6.1.1). Both were thin-walled and frequently branched and they lacked clamp connections but differences lay in hyphal diameters and in the frequency of septation. Hyphae of one type were very broad (8.0-10.0 µm in the advancing margin, and 8.0-12.0 µm in older parts) and sparsely septate; those of the other type were narrower (up to 8.0 µm) and much more frequently septate. The latter type was by far the more common and the following descriptions pertain to this type.

In the advancing margin, diameters of apical cells were 1.5-2.5 µm, and those of other cells, 2.0-3.5 µm. Septation was irregular. In narrower hyphae (1.5-2.0 µm), septa were widely separated with apical cells attaining lengths of up to 565 µm; in the broader hyphae (2.0-3.5 µm), septa were more frequent. In the unpigmented variant, hyphal diameters were double those recorded for the pigmented isolates (3.0-7.0 µm).

Behind the advancing margin, aerial mycelia were developed more profusely. In both aerial and appressed mycelia, hyphal diameters were 1.5-5.0 µm (3.5-10.0 µm in the unpigmented variant). Septation in the narrower aerial hyphae was similar to that in hyphae of the advancing margin. Septation in the broader aerial hyphae and all appressed mycelia was more frequent with cell lengths of up to 125 µm being recorded. In these latter hyphae, septa were present immediately distal to hyphal branches, and a septum was positioned in branches close to the hyphae bearing them (Fig. 6.1.2); these septa were usually absent in narrower hyphae of aerial mycelia and of the advancing margin (Fig. 6.1.3). Broader hyphae in all cultures had dolipore septa (Fig. 6.1.4).

In older parts of cultures, hyphal diameters were 1.5-8.0 µm (3.5-10.0 µm in the unpigmented variant). Narrower aerial hyphae were

Figure 6.1 Cytological features of mycelia from pigmented isolates and an unpigmented variant

1. Very broad, sparsely septate (bh) and narrower, more frequently septate hyphae (nh) produced in cultures of the fungus

2. Septa (s) in broader, frequently septate hyphae located immediately distal to branches (arrows depict direction to hyphal tips) and in branches, close to the hyphae bearing them

3. Narrower, less frequently septate hyphae. Note the absence of septa depicted in Fig. 2

4. Dolipore septa

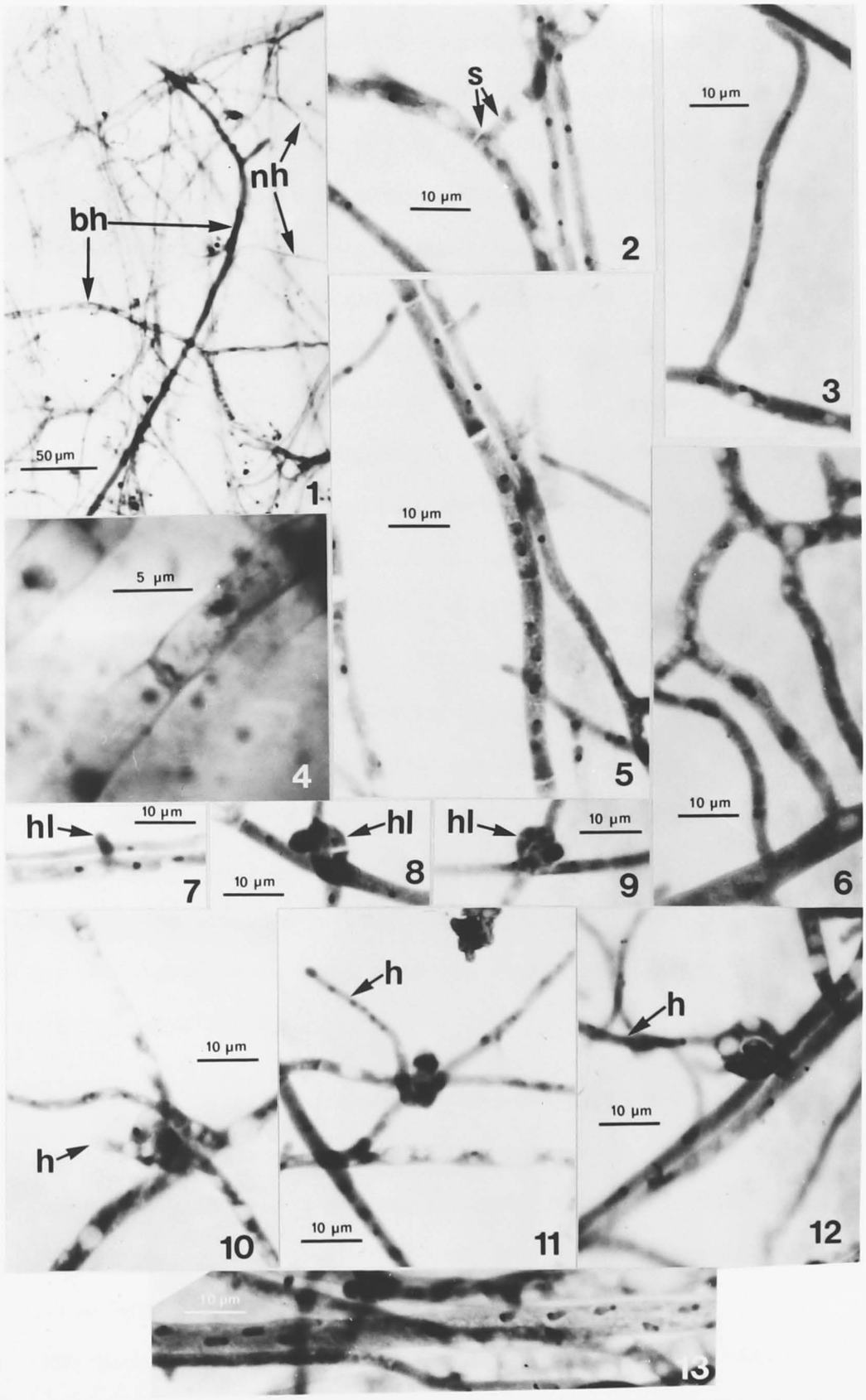
5. Short, broad cells in the older parts of cultures

6. Hyphal anastomoses

7-9. Advancing stages in the development of hyphal loops (hl)

10-12. New hyphae (h) arising from hyphal loops

13. Very broad, sparsely septate hypha (bh) containing numerous nuclei


bh
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50 µm
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s
10 µm
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4
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6
hl
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hl
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9
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10 µm
11
h
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12
10 µm
13

entirely converted into arthrospores. The frequency of septation increased with hyphal diameter and in the broadest hyphae (usually appressed to the cellophane), cells were 35-45 µm long (Fig. 6.1.5).

Hyphal anastomoses (Fig. 6.1.6) were observed in all cultures. They joined broad hyphae with other broad and narrower hyphae but were absent between narrower hyphae. Anastomosis was not observed in the advancing margin, but its frequency increased with distance from the margin. A structure, which may represent another mode of hyphal fusion and which was not seen previously in agar culture, occurred in cellophane cultures of pigmented isolates, but not in those of the unpigmented variant. Where two hyphae were in intimate contact, a short branch arose on one near the point of contact, and grew to form a loop encircling the other (Figs. 6.1.7-6.1.9). A single, new hypha then developed from each loop (Figs. 6.1.10-6.1.12). Loops of a hypha could encircle its own next order branch, or another hypha which originated some distance away. Where two hyphae lay together in intimate contact for any distance, more than one loop was often formed by one around the other. No unequivocal evidence of somatogamy was found, but it probably occurred as evidenced by the intimate contact between a loop and the encircled hypha, and the new hyphae which arose from the loops; these structures appear to serve no other function.

Nuclear Condition. Cells of very broad, sparsely septate hyphae contained numerous nuclei (Fig. 6.1.13), but because of their length and the interwoven nature of mycelium, accurate counts were rarely possible. At least 50 nuclei were counted in some cells. The remaining discussion relates to the more frequently septate hyphae.

The nuclear condition (number of nuclei per cell) of various cells of mycelia in cultures of the pigmented isolates and the unpigmented

variant is given in Table 6.1.

Table 6.1 Nuclear condition of various cells in hyphae of cultures of three pigmented isolates and an unpigmented variant of *P. noxius*

Isolate number	Nuclear condition (nuclei/cell)			
	Apical cells[1]	Subapical cells[1]	Older cells[2]	Arthrospores[2]
2250[3]	7.9 a a (2-26)	5.2 a b (2-15)	3.1 a c (1-9)	1.7 a d (0-5)
LB3[3]	7.3 a a (2-20)	5.5 a b (3-13)	2.4 b c (0-10)	1.9 b c (0-4)
LB3U[4]	7.0 a a (2-14)	4.8 a b (2-11)	2.5 b c (0-8)	2.4 c c (1-7)
LB5S[3]	8.1 a a (2-22)	5.1 a b (2-14)	3.3 a c (1-10)	1.6 a d (0-5)

[1]Cells of the advancing margin

[2]Cells of older parts

[3]Pigmented isolates

[4]Unpigmented variant

Figures in brackets are minima and maxima numbers of nuclei counted in the various cells

Values followed by the same letter do not differ significantly at p=0.05. The first letter pertains to vertical columns; the second, to horizontal rows

Mean numbers of nuclei in apical, subapical and older cells are determined from counts in 50 cells; those for arthrospores are from counts in 100 spores

The nuclear condition of corresponding cells in cultures of the north Queensland isolates (2250 and LB5S) were similar. In the advancing margin, apical cells contained significantly more nuclei (mean numbers of 7.9-8.1) than subapical cells (5.1-5.2). Cells of the oldest hyphae had 3.1-3.3 nuclei, significantly fewer than that recorded for subapical cells. Arthrospores had 1.6-1.7 nuclei, significantly fewer than cells of the older hyphae.

Numbers of nuclei in apical and subapical cells of cultures of the Malaysian isolate (LB3) were similar to those of the Queensland isolates but the older cells had fewer nuclei (a mean of 2.4). The isolate differed from the Queensland isolates also in the relative nuclear condition of older cells and arthrospores; both cell types of LB3 had similar nuclear counts (means of 2.4 and 1.9, respectively).

The nuclear condition of various cells in the unpigmented variant (LB3U) was similar to corresponding cells of the pigmented isolate from which it was derived, except that its arthrospores contained significantly more nuclei. However, as was the case for LB3, the number of nuclei in spores was similar to that of the older cells.

The results show that apical cells contain significantly more nuclei than subapical cells. This was often not the case in individual hyphal tips; many examples were encountered where an apical cell had fewer nuclei than the next cell. Also worthy of note is the wide variation in the number of nuclei among otherwise similar cells in the one culture. For example, the 50 apical cells of isolate 2250, in which counts were undertaken, had from two to 26 nuclei. Doubtless, apical cells with one, and more than 26 nuclei would be found in a larger sample.

6.2.3.2 Colonies Developing from Single Arthrospores of Isolate LB5S

Arthrospores are oblong to cylindrical. Spores in chains on the parent isolate were 2.5-30.0 µm long and 2.0-4.5 µm diam, and contained nil to five nuclei. Those harvested in suspension for this study were 2.5-15.0 µm long and 2.0-4.5 µm diam, and had one to three nuclei (Table 6.2; Fig. 6.2.1). In both instances, most spores were uninucleate.

Table 6.2 Percentage distribution of various numbers of nuclei in arthrospores of *P. noxius* in chains on cultures of the parent isolate, in spore suspension, and in chains on monoarthrospore cultures

Nuclei/spore	Percentage distribution[1]		
	Parent isolate	Spore suspension	Monosporous cultures
0	0.3±0.6	-	-
1	53.0±4.4	67.7±3.5	71.3±4.9
2	38.3±7.6	30.3±2.5	24.0±1.8
3	5.3±1.5	2.0±1.0	4.7±1.4
4	2.7±1.5	-	-
5	0.3±0.6	-	-
Mean nuclei/spore[2]	1.6 a	1.3 b	1.3 b

[1]Percentages were determined from three counts each of 100 spores

[2]Mean numbers of nuclei/spore were determined from 300 spores

Values of means followed by the same letter do not differ significantly at p=0.05

First germination was observed after 6 hr incubation; after 18 hr all spores had germinated (Table 6.3). A spore was considered to have germinated when the length of the germ tube equalled the diameter of the spore.

Table 6.3 Percent germination of arthrospores of *P. noxius* after various periods of incubation

Period of incubation (hr)	Percent germination[1]
0	0.0
3	0.0
6	3.0±2.0
12	86.3±4.2
18	100.0±0.0

[1]Percent germination was determined from three counts, each of 100 spores

Figure 6.2 Cytological features of the germination of arthrospores and of the development of single spore colonies

1. Arthrospores are uni-, bi-, or multi-nucleate

2. A small protuberance develops on the spore wall adjacent to a nucleus at the onset of germination

3. The nucleus migrates to the protuberance and remains partly in the spore while a germ tube develops

4. The nucleus migrates up the elongating germ tube after the tube has attained a length of c.3µm

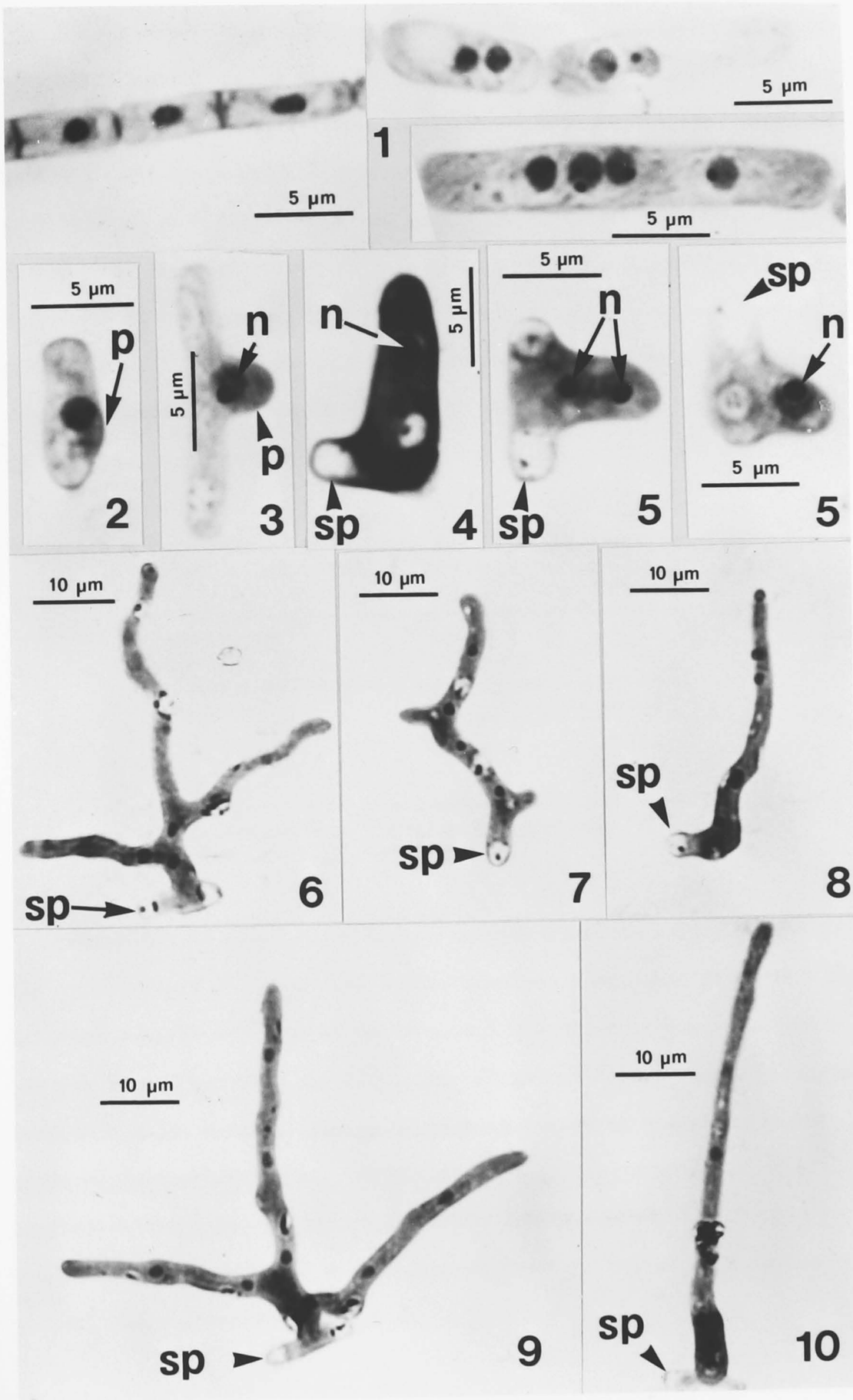
5. Spores are devoid of nuclei before or shortly after they have germinated

6-8. Germ tubes may grow from the side (Fig. 6), end (Fig.7), or rim (Fig. 8) of the spore

9. Germination hyphae usually branch within a short distance of the spore

10. Some germination hyphae remain unbranched until they grow to considerable lengths

(n = nucleus; p = protuberance; sp = spore)


5 µm
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7
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sp
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10

At the onset of germination, a small protuberance developed on the spore wall adjacent to a nucleus. The nucleus migrated to the protuberance as soon as the latter began to develop (Fig. 6.2.2), and remained partly in the spore while the protuberance elongated apically into a germ tube (Fig. 6.2.3). When the germ tube was about 3 µm long, the nucleus commenced migrating up the tube as the tube lengthened (Fig. 6.2.4). Nuclei migrated from spores during or shortly after germination (Fig. 6.2.5). Each spore produced only a single germ tube which grew usually from the side, but occasionally from the end or rim of the spore (Figs. 6.2.6-6.2.8; Table 6.4).

Table 6.4 Percentage of germ tubes growing from the side, end or rim of arthrospores of *P. noxius*

Location of germ tube	Percent[1]
Side	73.0±4.6
End	13.7±2.5
Rim	13.3±2.3

[1]Percentages were determined from three counts, each of 100 spores

Branching of germination hyphae usually occurred within 6.0 µm of the spore (Fig. 6.2.9), but some hyphae remained unbranched until they had attained lengths of up to 50 µm (Fig. 6.2.10). At least two nuclei were present in all branched colonies, and unbranched hyphae never contained more than eight nuclei. During subsequent growth of colonies, hyphae produced frequent branches. Branches generally arose adjacent to a nucleus and were usually of considerable length (up to 15.6 µm) before the nucleus migrated into it. Distances from the hyphal apex to the closest nucleus varied from 8.5 µm to 17.0 µm.

First septation was observed in colonies after 18 hr incubation. The number of nuclei present in a colony when the first septum formed varied considerably. A minimum of six nuclei (three in each of two cells) was found in septate colonies, and a maximum of 30 nuclei was observed in aseptate colonies.

The number of nuclei in cells of monosporous colonies varied widely; it varied with the age of a colony and the location of a cell in a colony, and also between cells in the one colony (Table 6.5). The average number of nuclei increased progressively from 1.3 in arthrospores in fresh suspension (0 hr incubation) to 9.6 in apical cells of colonies 24-hr old; no further increase occurred in the number of nuclei in apical cells with time.

Table 6.5 Nuclear condition of various cells in hyphae of colonies arising from single arthrospores of *P. noxius* after different incubation periods

Period of incubation (hr)	Nuclear condition (nuclei/cell)			
	Apical cells[1]	Subapical cells[1]	Older cells[2]	Arthrospores[2]
0	-	-	-	1.3 a - (1-3)
18	6.1 a a (1-14)	3.0 a b (3-3)	-	-
24	9.6 b a (3-25)	7.6 b b (2-24)	-	-
96	10.2 b a (1-29)	5.1 c b (1-11)	2.4 a c (1-9)	1.3 a d (1-3)
Parent isolate	8.1 c a (2-25)	5.1 c b (2-14)	3.3 b c (1-10)	1.6 b d (0-5)

[1]Cells of the advancing margin

[2]Cells of older parts

Figures in brackets are minima and maxima numbers of nuclei counted in the various cells

Values followed by the same letter do not differ significantly at p=0.05. The first letter pertains to vertical columns; the second to horizontal rows

Mean numbers in arthrospore colonies were determined from counts of 300 spores, and 100 each of other cells (except for subapical cells at 18 hr, where only two subapical cells were seen), and in the parent isolate, from 100 spores, and 50 each of other cells

While the number of nuclei in apical cells were similar in colonies incubated for 24 hr and 96 hr, the subapical cells of the older (96 hr) colonies contained fewer nuclei than those of the younger (24 hr) colonies. In the younger colonies, many hyphal branches in the advancing margin had only a single septum, so subapical cells often included nuclei in hyphae giving rise to the branches. Additional septa were formed in the branches of the older colonies and subapical cells were separate from the hypha bearing the branch, and so had fewer nuclei.

The mean number of nuclei in various cells of 96 hr old monosporous colonies differed in most respects from that of the parent isolate: their apical cells contained significantly more nuclei, and their older cells and arthrospores, significantly fewer. Only subapical cells of both had a similar number of nuclei.

Arthrospores produced by monoarthrospore colonies had the same number of nuclei as the arthrospores comprising the inoculum for the colonies. Both differ from that of the parent (LB5S) from which the inoculum was prepared, in both the mean number of nuclei and the percentage distribution of uni-, bi- and multi-nucleate spores (see also Table 6.2). Over 70% of the arthrospores of monoarthrospore colonies had only a single nucleus while in LB5S, just over 50% were uninucleate.

Diameters of germ tubes and of hyphae in colonies incubated for up to 24 hr were 3.0-3.5 µm. Septa in these colonies were simple. No hyphal fusions were observed either within or between colonies.

After 48 hr incubation, the diameter range increased to 2.0-5.0 µm. Dolipore septa were found in the older, broader hyphae. Rare hyphal anastomoses occurred between hyphae of the same colony but not between hyphae of different colonies. Hyphal loops, as described previously for pigmented isolates from basidiocarps and host tissues, were frequently

seen formed by hyphae of one colony around hyphae of the same or different colonies.

The micromorphology of monoarthrospore colonies incubated for 96 hr was similar to that of pigmented isolates.

6.2.3.3 Colonies Developing from Single Basidiospores of Isolate LB5S

Basidiospores harvested in suspension were ellipsoid with lengths of 4.0-5.0 µm and maximum width of 3.0-4.0 µm. They contained nil to three nuclei (Figs. 6.3.1-6.3.4) but spores with three nuclei were rare (Table 6.6).

Table 6.6 Percentage distribution of various numbers of nuclei in basidiospores of *P. noxius*

Nuclei per spore	Percentage[1] distribution
0	38.3±2.5
1	49.1±4.2
2	12.3±1.6
3	0.3±0.5
$\bar{X}$ [2]	0.7

[1]Percentages were determined from three counts, each of 100 spores

[2]The mean value was determined from 300 spores

Almost 80% of nucleated spores contained a single nucleus. The result for mean nuclei per spore (0.7) was surprising. Four nuclei (or eight, if a post-meiosis mitosis takes place) presumably occur in a basidium following meiosis. If all nuclei migrate to basidiospores, a value close to 1.0 (or 2.0 if eight nuclei are in each basidium) would be expected.

Figure 6.3 Cytological features of the germination of basidiospores and of the development of single spore colonies

1-4. Basidiospores may be anucleate (1), or contain 1(2), 2(3), or rarely, 3(4) nuclei

5. At the onset of germination, a protuberance develops at the apex of the spore

6. The protuberance expands to form a vesicle of dimensions similar to those of the spore and which is delimited from the spore by a constriction

7-8. Either one (7) or two (8) hyphae arise from the vesicle

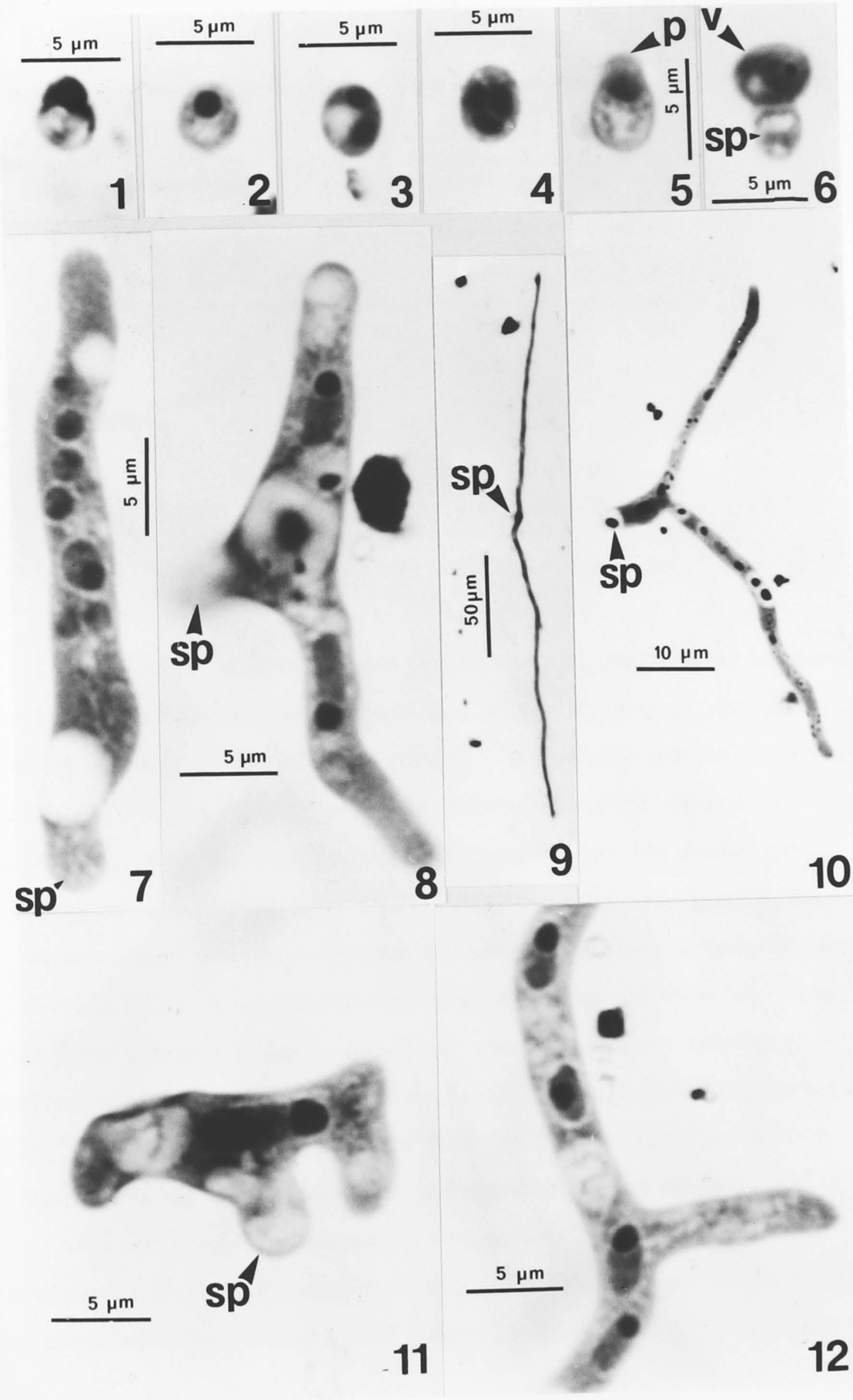
9. Where two hyphae arise from the vesicle, they grow in opposite directions for some distance

10. Subsequent branching can take place close to the vesicle

11. Some young branched colonies contain only a single nucleus

12. Branches arise adjacent to a nucleus and grow to considerable lengths before nuclei migrate into them

(p = protuberance; sp = spore; v = vesicle)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8
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50µm
9
sp
10 µm
10
5 µm
sp
11
5 µm
12

First germination occurred after 12 hr incubation; after 48 hr, ungerminated nucleated spores were not observed (Table 6.7).

Table 6.7 Percent germination of nucleated basidiospores of *P. noxius* after various periods of incubation

Period of incubation (hr)	Percent[1] germination
0	0.0
6	0.0
12	2.7±0.5
18	22.7±2.1
24	67.3±1.2
48	100.0±0.0

[1]Percent germination was determined from three counts, each of 100 nucleated spores

At the onset of basidiospore germination, a protuberance developed at the apex of the spore (Fig. 6.3.5). Nuclei migrated to the vicinity of the protuberance as it was initiated. The protuberance then expanded to form a vesicle which attained dimensions similar to those of the spore, and which was delimited from the spore by a constriction (Fig. 6.3.6); nuclei migrated into the vesicle during its development. Spores were considered to have germinated when the vesicle was as long as the spore was wide. One or two hyphae then arose from the vesicle. If only one hypha was produced, its subsequent linear growth was initially, approximately along the spore-vesicle axis (Fig. 6.3.7); where two hyphae grew from the vesicle, they did so from opposite sides of the vesicle (Fig. 6.3.8) and continued to grow in opposite directions (Fig. 6.3.9).

Most colonies branched either at the vesicle, or within a short distance of it (Fig. 6.3.10), but some germination hyphae remained unbranched until they had attained lengths of up to 156 µm. Some young

branched colonies contained only a single nucleus (Fig. 6.3.11). A maximum of eight nuclei were found in unbranched colonies. Branches in both young and older colonies usually grew adjacent to a nucleus and were generally of considerable length (up to 34 µm) before the nucleus migrated into it (Fig. 6.3.12). Where nuclei were present in branches, distances from the hyphal apex to the closest nucleus varied from 8.5 µm to 17.0 µm.

Septation was first seen in colonies after 24 hr incubation. Colonies varied considerably in the number of nuclei they contained when the first septum was formed; a minimum of two nuclei (one in each of two cells) was found in a septate colony, but some aseptate colonies had as many as 30 nuclei. The first septum formed was almost uniformly at the base of a branch, and so some apical cells were very long.

Hyphae in colonies incubated for up to 24 hr were 2.5-4.0 µm diam, and had widely separated, simple septa. Hyphae were broader (2.0-6.0 µm diam) after 48 hr incubation, and septa were more frequent, especially in older parts; some dolipore septa had formed in broader hyphae by this time. After 96 hr incubation, hyphae were 1.5-2.9 µm diam in the advancing margin and 1.5-11.0 µm in the older parts; narrower aerial hyphae were converted into arthrospores. Septation in older colonies was variable; some colonies had frequent septa, even in the advancing margin. However, in most colonies, septa were so positioned that a penultimate cell in a hypha nearly always bore an aseptate branch, and so the penultimate cell itself was part of an apical cell. Hence, only limited data was obtained on the nuclear condition of subapical cells.

Nucleated basidiospores contained an average 1.2 nuclei. After 24 hr incubation, when septation first occurred, apical cells in the advancing margin of colonies had an average of 10.0 nuclei; no further

significant increase, or decrease, in this number occurred with time (Table 6.8). All the various cells (apical, older and arthrospore) of monosporous colonies after 96 hr incubation, contained significantly more nuclei than the corresponding cells of the parent isolate.

Table 6.8 Nuclear condition of various cells in hyphae of colonies developing from single basidiospores of *P. noxius* after different periods of incubation

Period of incubation (hr)	Nuclear condition (nuclei/cell)		
	Apical cells[1]	Older cells[2]	Arthrospores[2]
0			1.2[3] (1-3)
24	10.0 a - (1-29)	-	-
48	10.6 a - (1-32)	-	-
96	11.1 a a (1-46)	4.0 a b (1-17)	2.0 b c (0-6)
Parent isolate	8.1 b a (2-25)	3.3 b b (1-10)	1.6 c c (0-5)

[1]Cells of the advancing margin

[1]Cells of older parts

[3]Mean nuclei in basidiospore inoculum

Figures in brackets are minima and maxima numbers of nuclei found in the various cells

Values followed by the same letter do not differ significantly at p=0.05. The first letter pertains to vertical columns; the second to horizontal rows

Mean values in arthrospore colonies were determined from counts of 300 spores, and 100 each of other cells, and in the parent isolate, from 100 spores, and 50 each of other cells

Hyphal loops, as described for pigmented isolates from basidiocarp and host tissues, were observed in colonies incubated for 48 hr. However, not all colonies formed them. Where they were formed, they encircled other hyphae of the same colony and also hyphae of other colonies.

During the above studies, differences were noted among monosporous colonies in their cytology. For example, apical cells of some colonies contained more nuclei than others, there were differences in hyphal diameters, and some colonies formed hyphal loops while others did not. Ten monosporous isolates used in previous studies were grown on cellophane for 96 hr and the nuclei were stained. Six of them, representing three morphological types, were selected for detailed cytological studies.

There appeared to be no correlation between hyphal diameter and cultural type. For example, the broadest hyphae in isolates with white, appressed cultures varied from 5.5 µm to 11.0 µm, and those in isolates with intermediate-type cultures (mid-brown, with a white aerial bloom) varied from 4.5 µm to 9.0 µm. However, the broadest hyphae in isolates with uniformly dark brown, appressed cultures were all 7.5 µm.

The formation of hyphal loops was correlated with morphology. All four isolates with white appressed cultures formed these structures, whereas they were absent in cultures of the other six isolates representing the other two morphological types.

The number of nuclei in cells of cultures of the various basidiospore isolates is also correlated with cultural morphology (Table 6.9). The apical cells and arthrospores of the white appressed isolates contain approximately the same number of nuclei as the corresponding cells of the dark brown, appressed isolates. However, isolates with the intermediate cultural morphology have approximately twice the number of nuclei per cell of the other two.

Table 6.9 Nuclear condition of various cells in hyphae of cultures of monobasidiospore isolates of *P. noxius* with different cultural morphology

Cultural[3] type	Isolate number	Nuclear condition (nuclei/cell)		
		Apical cells[1]	Older cells[2]	Arthrospores[2]
I	B4	7.4 a b (1-29)	2.7 a (1-7)	1.5 a (1-3)
	B12	9.1 a (1-23)	3.1 a b (1-7)	1.9 b (1-4)
II	B9	8.5 a (3-27)	4.0 b (1-8)	1.5 a (1-4)
	B17	6.2 b (2-20)	3.5 b (1-9)	1.2 c (0-3)
III	B20	16.0 c (2-46)	5.3 c (1-15)	2.4 d (1-5)
	B28	15.7 c (1-40)	5.5 c (1-17)	2.8 e (1-6)

[1]Cells of advancing margin

[2]Cells of older parts

[3]Cultural types. I: Uniformly dark brown, appressed; II: White, appressed; III: Intermediate (mid-brown with white aerial bloom)

Figures in brackets are minima and maxima numbers of nuclei found in the various cells

Values followed by the same letter do not differ significantly at p=0.05. Letters refer only to vertical columns; in all cases, differences between the various cells were significant

Mean values in each case were determined from 100 spores, and 50 each of the other cells

6.2.4 Discussion

The nuclear condition in *P. noxius* is quite different from that of "textbook" Hymenomycetes such as *Clitocybe truncicola* (Bistis, 1970), *Fomes cajanderi* (Neuhauser & Gilbertson, 1971), *Collybia velutipes* (Curt. ex Fr.) Kummer, *Coprinus* sp., *Polyporus betulinus* Bull. ex Fr. and *Schizophyllum commune* (Raper, 1966). Cells are multinucleate, septal clamp connections are absent, and there is no evidence of nuclear pairing

or synchronous division. Most mature arthrospores and basidiospores are uninucleate or binucleate, but the multinucleate condition of tissue isolates is regained on cellophane membrane within 12-18 hr.

Phellinus noxius is placed in the Hymenochaetaceae (Fidalgo, 1968). The "diplont" mycelia of *Phellinus* and other species in the family comprise two groups separated on nuclear condition: those with two or three nuclei per cell; and those with multinucleate cells (Boidin, 1971). *Phellinus noxius* fits the latter pattern, together with *Phellinus conchatus* (Pers. ex Fr.) Quél.and *Phellinus igniarius*. Species of *Phellinus* conforming to the former pattern include *P. gilvus, P. nigrolimitatus* (Romell) Egel., and *P. torulosus* (Pers. per Pers.) Quél. (Boidin, 1971), and *P. weirii* (Hansen, 1979a).

The variable nuclear condition of basidiospores of *P. noxius* is unusual, though not unique, among the Holobasidiomycetidae. All 395 species of Aphyllophorales analysed by Boidin (1971), and 41 species of holobasidiomycetes investigated by Duncan and Galbraith (1972) produced spores which were consistently uninucleate or binucleate. However, variation in nuclei per cell has been observed in other Holobasidiomycetidae such as *Agaricus bisporus* Lange (Imbach) (Lemke *et al.*, 1975), *Phellinus weirii* (Hansen, 1979a), and *Volvariella volvacea* (Bull. ex Fr.) Sing. (Chang & Ling, 1970).

Variable nuclear condition among monobasidiosporous isolates has not been reported previously. Nuclear condition was correlated with cultural features of isolates: apical cells of the two appressed forms (white and uniform dark brown) contained half the number of nuclei in those of the intermediate (mid-brown, with a white aerial bloom) form. The older cells of the former also had half to two-thirds the nuclei found in the latter. These results suggest that intermediate isolates came from

binucleate spores, and represent the heterokaryons, and that the two appressed forms came from uninucleate spores, and are homokaryons. However, apical cells of the presumed heterokaryons from host and basidiocarp tissues had only half the number of nuclei of the intermediate isolates, and a statistically similar number to that of the appressed isolates. The role of all three forms in the life cycle of *P. noxius* requires elucidation.

The function of hyphal loops also requires elucidation. These structures have not been recorded in other Holobasidiomycetidae in the literature. Microscopical observations, which were not unequivocal, strongly suggest a mode of hyphal fusion.

Monobasidiosporous and heterokaryotic isolates must be distinguished when interpreting reactions in tests for sexual incompatibility. Criteria which have allowed separation in other Holobasidiomycetidae include the presence of septal clamp connections on heterokaryotic mycelia (Raper, 1966; Adams & Roth, 1967; Korhonen, 1978b); differences between homokaryons and heterokaryons in colony morphology (Korhonen, 1978a; Raper & Kaye, 1978); or the formation of fertile basidiocarps by the heterokaryon (Raper, 1966). Recently, nuclear counts, either alone or in combination with colony morphology, have been used with multinucleate species (Raper & Kaye, 1978; Hansen, 1979a). None of the above appear useful for *P. noxius*; other criteria, for example interaction at the common margin of paired isolates (Hansen, 1979b) need investigation.

6.3 EXPERIMENT 2: NUCLEAR BEHAVIOUR IN BASIDIA AND VEGETATIVE MYCELIA

6.3.1 Introduction

In the previous experiment (Section 6.2), it was shown that cells of isolates of *P. noxius* from basidiocarp or host tissues are multinucleate, but the number of nuclei in similar cells of the one culture varies widely. For example, apical cells in the advancing margin of a culture of isolate LB5S contained two to 25 nuclei. Basidiospores of the fruiting body from which LB5S was isolated, contained one to three nuclei but developed colonies which had one to 46 nuclei in their apical cells. Similarly, arthrospores of isolate LB5S containing one to three nuclei, gave rise to colonies in which apical cells had two to 26 nuclei. Most spores of both types were uninucleate. Also apical cells frequently had fewer nuclei than their subapical cells.

The above results pose questions on the pre- and post-meiotic events which take place in basidiocarps of the fungus and also on the mode and synchrony of nuclear division, and on nuclear migration in vegetative hyphae. These phenomena were investigated in the following experiment.

6.3.2 Materials and Methods

Material for studies on basidia came from several field collections of sporulating basidiocarps and also from the basidiocarp which provided some of the material used in the previous experiment.

Nuclei were stained with Meyer's haemalum and Giemsa stain. Slides prepared for the previous experiment were used for observations on haemalum-stained nuclei in vegetative mycelia.

For Giemsa-staining, an additional cellophane strip was cut from cultures of the previous experiment. Strips were placed in a 6:1:1 mixture of absolute ethanol, glacial acetic acid and lactic acid for 10 min, then passed through 95% ethanol (for 1 min) to 70% ethanol. Material was either stored in the ethanol, or if the process was to be continuous, washed for 10 minutes in it. Strips were then rinsed in distilled water, hydrolysed in 5N HCl at room temperature for 40 min, washed for 5 min in distilled water, and placed in phosphate buffer (pH 7.2: 0.2% Na_2HPO_4 was added to 0.2% KH_2PO_4 until the desired pH was reached; both solutions were w/v) for 5 min. Nuclei were stained for 30 min in a fresh Giemsa solution containing 1.0 ml Gurr's improved R66 Giemsa stain and 10.0 ml phosphate buffer. The cellophane and cytoplasm were cleared by immersion for 6 min in a 0.25% solution of trypsin in phosphate buffer. Finally the material was washed for 5 min in phosphate buffer and mounted in a 1:1 mixture of glycerol and buffer. Coverslips were sealed with cosmetic nail varnish.

Similar staining procedures were used on thin sections from basidiocarps.

Slides were examined with a Carl Zeiss photomicroscope using bright field optics and a green filter. Film used was either Kodak Panatomic X or Agfa Copex Pan rapid.

6.3.3 Results

6.3.3.1 Nuclear Behaviour in Basidia

Repeated attempts were made to stain nuclei in basidia and attached basidiospores; these attempts involved extensive experimentation, particularly with the more flexible Giemsa-staining, using various procedures and periods for fixation, hydrolysis and staining, on material

from a number of basidiocarp collections. However, all were unsuccessful.

Examination of material with a scanning electron microscope revealed that the hymenium lining the pores of a basidiocarp is coated with a mucilage (Fig. 6.4.1). When sporulation takes place, sterigmata penetrate through the mucilage (Fig. 6.4.2) and spores develop above it (Fig. 6.4.3) but are coated with a thin mucilaginous layer (Fig. 6.4.4). Staining of nuclei in attached basidiospores and basidia might well be impeded by this material. When basidiospores are collected overnight in water, nuclei are readily stained by either of the techniques described above. This suggested that soaking thin sections from basidiocarps in water might facilitate staining, but when tried, no improvement occurred.

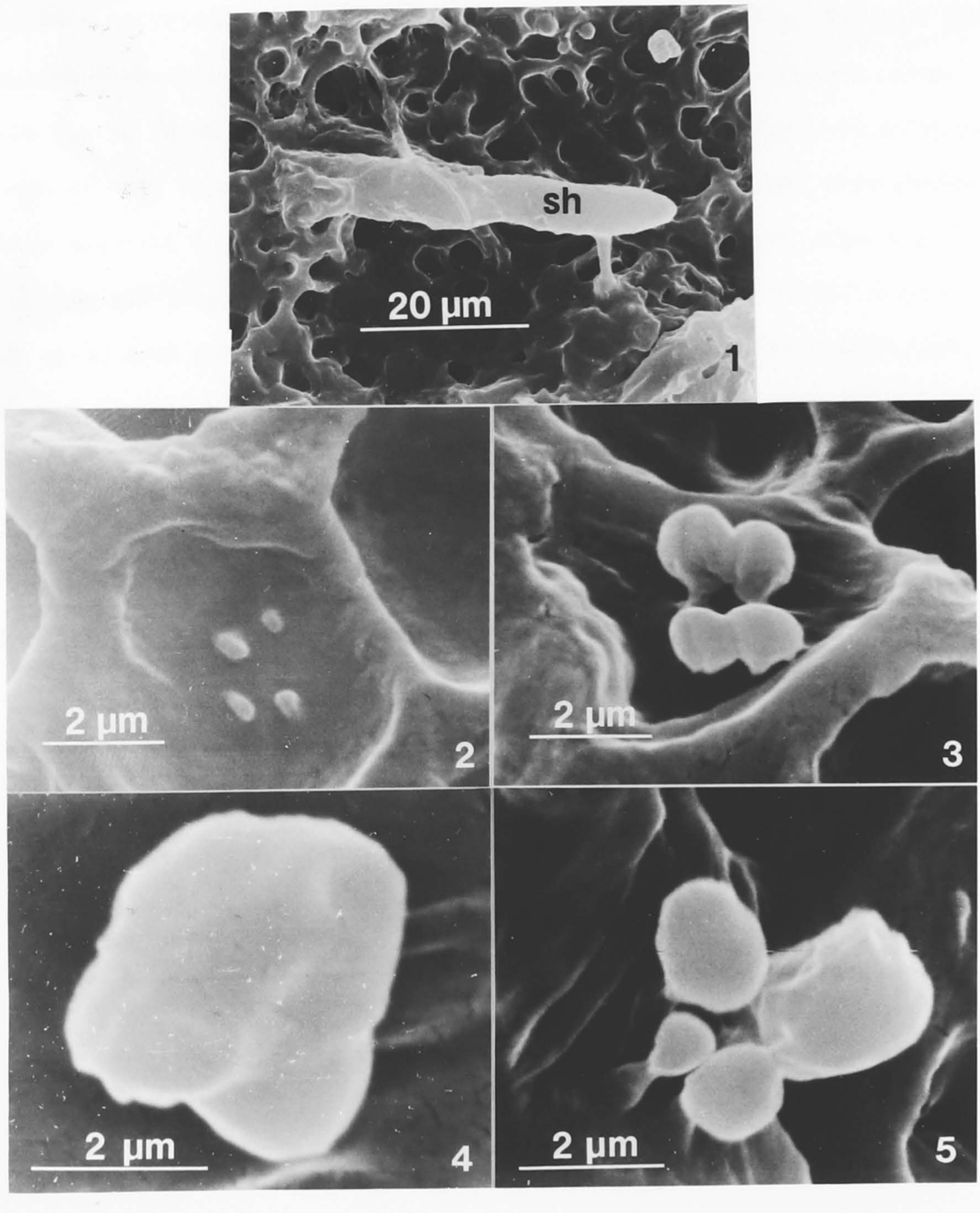
Hence, post-meiotic events in *P. noxius* were not clarified in these studies. In most tetra-spored Polyporaceae, four post-meiotic nuclei occur in the basidium and they migrate to separate spores, so that each spore, initially at least, has a single nucleus. However, a high percentage of basidiospores of *P. noxius* (about 38% in the previous experiment - Table 6.6) were anucleate, and about thirteen per cent had more than one nucleus. These data can be reconciled with spore development as observed with the scanning electron microscope. Frequently, spores on the one basidium develop at different rates (Fig. 6.4.5). It is therefore possible that more than one nucleus can migrate into the larger spores, leaving at least one spore of the tetrad devoid of nuclei. The latter, however, do not abort but continue to enlarge so that all spores on the basidium are approximately of equal size at maturity. This mechanism would explain the occurrence of a-, bi-, and tri-nucleate spores.

6.3.3.2 Nuclear Behaviour in Vegetative Mycelia

Nuclei were of two basic shapes; stationary nuclei were spherical, or nearly so, whereas migrating and dividing nuclei were elongated along

Figure 6.4 Spore development on basidia

1. The hymenium lining pores is coated with mucilage (sh = setal hyphae which *inter alia*, are characteristic of basidiocarps of *P. noxius*)
2. When sterigmata develop, they penetrate through the mucilage
3. Spores develop above the mucilage coating
4. Spores often have a thin coating of mucilage which binds them together
5. Developing spores frequently differ in size, although eventually attaining similar dimensions


sh
20 µm
1
2 µm
2
2 µm
3
2 µm
4
2 µm
5

the hypha.

Stationary nuclei stained uniformly and intensely with Giemsa whereas those treated with Meyer's haemalum displayed an intensely stained nucleolus in a less intensely stained nucleus (Fig. 6.5.1). All nuclei in basidiospores, arthrospores and young monoarthrospore colonies (up to 18 hr old) and most nuclei in young monobasidiospore colonies, were of this type. Nearly all nuclei in hyphae of isolates from basidiocarp and host tissues, and older monosporous colonies were migrating.

In all fungal material examined, stationary nuclei varied widely in size, even within the one cell; diameters of the nuclei ranged from 1.0 μm to 2.5 μm. Variation in nuclear size in arthrospores and basidiospores of isolate LB5S is demonstrated in Figs. 6.5.2 and 6.5.3. Also, the nuclei in hyphae and arthrospores of the unpigmented variant LB3U appeared to be significantly larger than those of its pigmented counterpart (Figs. 6.5.4 and 6.5.5).

Migrating nuclei stained with Giemsa were tear-shaped and had an intensely stained centriole at the tip of its tail (Fig. 6.5.6); nuclei were granular in appearance. Those stained with haemalum were elongated with a prominent nucleolus at one end (Fig. 6.5.7). The nucleolus leads the nucleus in migration (Figs. 6.5.6 and 6.5.7). Occasionally, nuclei were observed passing through septa.

Nuclear division did not follow classical mitosis, and nuclear bodies which could be recognized unequivocally as chromosomes were not observed. Before division, nuclei rounded up; nucleoli and centrioles were prominent at this stage (Fig. 6.6.1). The nucleus then began to elongate and while this occurred, the nucleolus was displaced into the cytoplasm (Figs. 6.6.2 and 6.6.3), the centriole enlarged and divided, and one of the daughter organelles migrated to the opposite end of the

Figure 6.5 Cytology of nuclei in vegetative hyphae

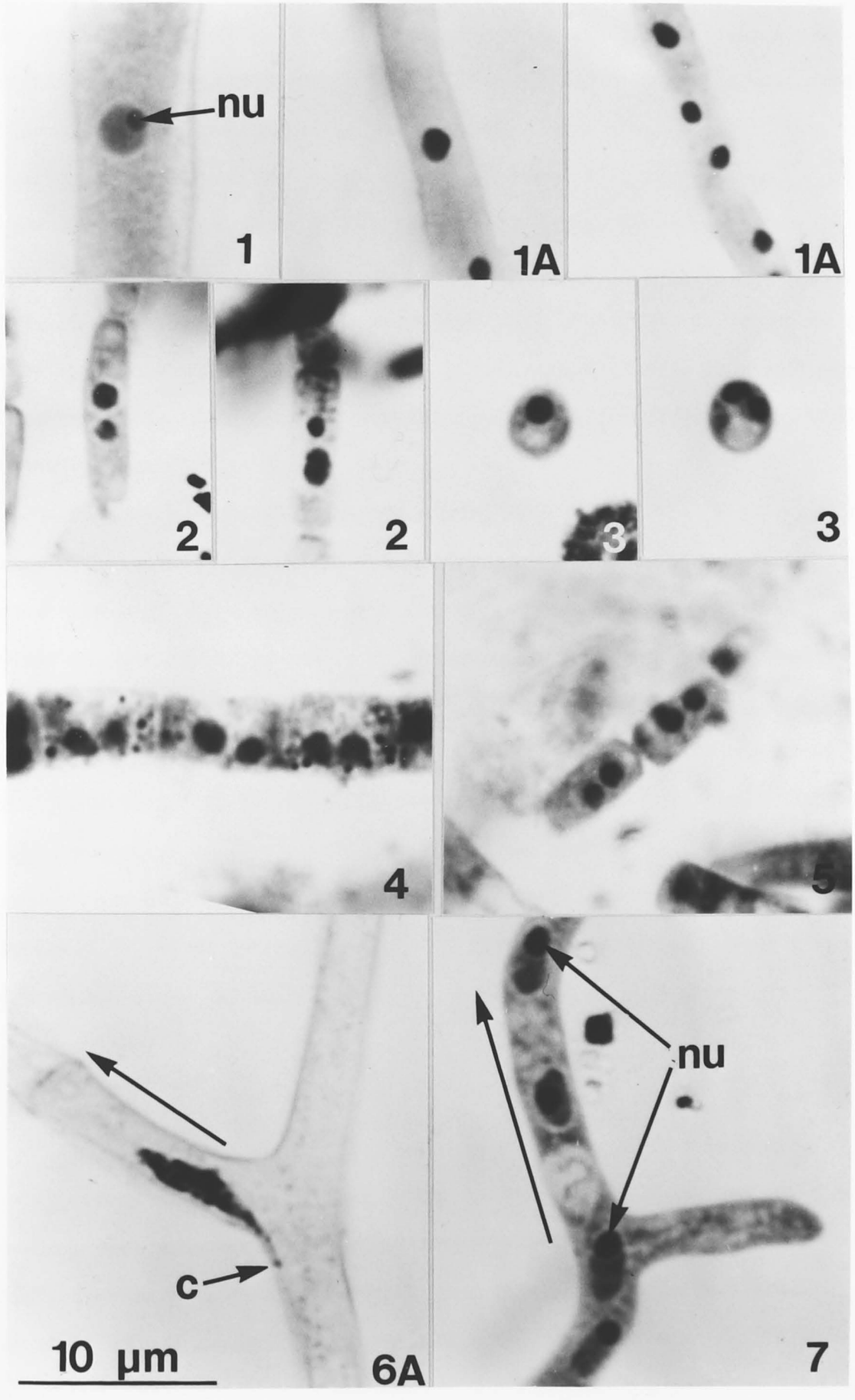
1. Stationary nuclei. Giemsa-stained nuclei are uniformly and intensely stained. A nucleolus (nu) is prominent in nuclei stained with Meyer's haemalum

2-3. Stationary nuclei vary widely in size. These figures depict variation in size of nuclei within the one arthrospore (Fig. 2), and among basidiospores (Fig. 3)

4-5. Nuclei of the unpigmented variant (LB3U) are larger than those of its pigmented counterpart (LB3). Nuclei in arthrospores of LB3U are depicted in Fig. 4, and those of LB3, in Fig. 5

6-7. Migrating nuclei. Giemsa-stained nuclei are tear-shaped and granular, and had an intensely stained centriole (c) at the end of its tail. Those stained with haemalum were elongated and had a prominent nucleolus (nu) at its leading end. Arrows depict movement towards the hyphal apex

Figures followed by the letter 'A' depict Giemsa-stained nuclei; the remainder were stained with Meyer's haemalum


nu
1
1A
1A
2
2
3
3
4
5
c
10 µm
6A
nu
7

nucleus (Figs. 6.6.4 and 6.6.8). On rare occasions, the nucleolus was retained by the nucleus (Fig. 6.6.9). The nucleus then divided with the line of separation at about 45° to the hyphal axis (Fig. 6.6.10), but occasionally, it was transverse across the hypha (Fig. 6.6.11) or parallel to the hyphal axis (Fig.6.6.12). Finally, the daughter nuclei moved apart and either rounded up a short distance from each other (Fig. 6.6.13) or continued to migrate away from each other. Nuclei appeared to vary in chromatin content (Figs. 6.6.14 to 6.6.16) and in some instances, daughter nuclei of the one parent nucleus appeared to differ in chromatin content (Figs. 6.6.12 to 6.6.16).

The frequency of dividing nuclei was very low (<1%). This suggested that division might be almost synchronous, very rapid, or that the fixing solutions might have allowed most dividing nuclei to complete their division, but inhibited the initiation of division. However, division is probably not synchronous in the fungus, even in young monosporous colonies (Fig. 6.6.17). Differences were noted in the frequency of division, between nuclei fixed by the two methods. The "double track" configuration characteristic of dividing nuclei fixed and stained by the Giemsa method were more frequent than replicating centrioles and separating daughter nuclei in material stained by the haemalum method. Thus the fixing solutions may have allowed the completion of most divisions.

6.3.4 Discussion

Basidiola of the Holobasidiomycetidae are initially binucleate. During their development into basidia, the two nuclei fuse, and the fusion nucleus then undergoes meiotic division. Sterigmata begin to emerge from the apices of basidia at prophase I to telophase II of meiosis (Wilson *et al.*, 1967; Tommerup & Broadbent, 1975; Restivo & Petersen, 1976).

Figure 6.6 Nuclear division in vegetative mycelia.

1. Before division, nuclei round up. The nucleolus (nu) and centriole (c) are prominent.

2-3. The nucleolus (nu) is displaced into the cytoplasm.

4-8. The centriole enlarges and divides, and daughter centrioles (c) move to opposite ends of the nucleus. The nucleolus has dissipated and is no longer visible.

9. The nucleolus (nu) is retained on occasions.

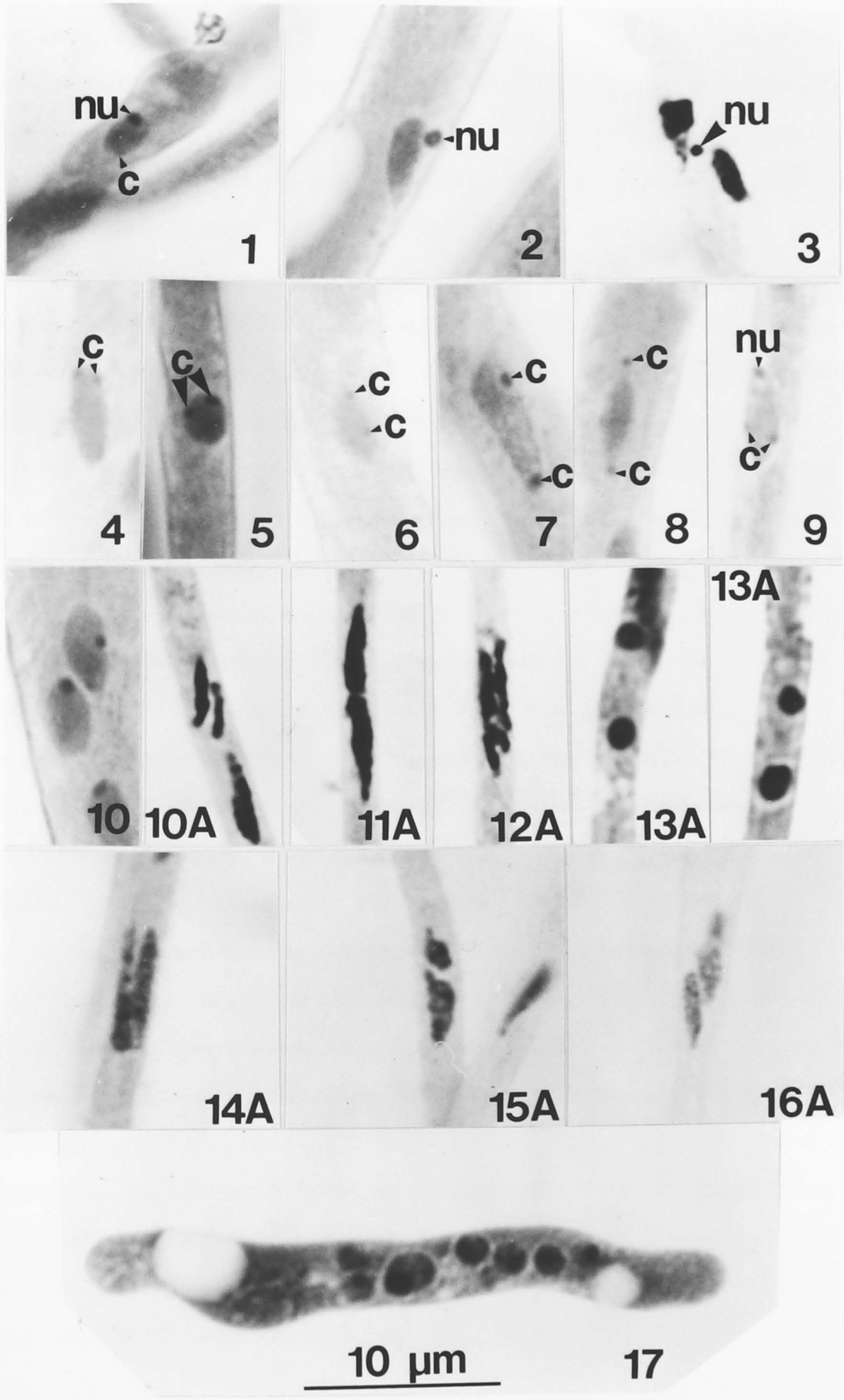
10-12. The nucleus physically separates with the line of separation usually at 45^{o} to the longitudinal axis of the hypha (Fig. 10), but it may separate with the line transverse across the hypha (Fig.11), or parallel to it (Fig.12).

13. Daughter nuclei move away from each other and often round up.

14-16. Nuclei appear to vary in chromatin content.

17. Division is not synchronous. This is evident even in very young colonies.

Figures followed by the letter 'A' depict Giemsa-stained nuclei; the remainder were stained with Meyer's haemalum.


nu
c
1
nu
2
nu
3
c
4
c
5
c
c
6
c
c
7
c
8
nu
c
9
10
10A
11A
12A
13A
13A
14A
15A
16A
10 µm
17

Following meiosis, basidia contain four nuclei. There are conflicting accounts on post-meiotic events in the sub-class. Duncan and Galbraith (1972) disclaim the generalization that four post-meiotic nuclei migrate into four basidiospores which are discharged in an uninucleate condition; they confidently state from their investigations into 41 species, that a third nuclear division is a regular feature. Other authors have reported the occurrence of the third division in fungi such as *Agaricus bisporus* (Raper & Raper, 1972; Lemke *et al.*, 1975), *Armillariella mellea* (Tommerup & Broadbent, 1975), and *Heterobasidion annosum* (Wilson *et al.*, 1967). However, literature appearing after that of Duncan and Galbraith (1972) contains examples of species where there is no post-meiotic mitosis; these include *Lentinus edodes* (Berk.) Sing. (Nakai & Ushiyama, 1978), *Marasmius perniciosus* Stahel (Delgardo & Cook, 1976), *Phellinus weirii* (Hansen, 1979a) and *Poria latemarginatus* (Dur. et Mont.) Cke. (Setliff *et al.*, 1974). Data from the present studies do not give evidence for a mitotic division following meiosis, but *P. noxius* certainly does not fit the pattern D of Duncan and Galbraith (1972). In that pattern, the third division takes place in the spore and both daughter nuclei remain in the spore, which is discharged as binucleate. Almost 80% of harvested nucleated spores of *P. noxius* and many germlings were uninucleate.

Variable nuclear condition among basidiospores has been reported for other Holobasidiomycetidae. Basidiospores of *P. weirii* and *Volvariella volvacea* are usually uninucleate but a small percentage are binucleate (Chang & Ling, 1970; Hansen, 1979a). The binucleate condition in the latter was attributed to precocious karyofission in the spore prior to germination (Chang & Ling, 1970). Hansen (1979a) did not comment on the origin of binucleate spores of *P. weirii*, but the explanation of Chang and Ling (1970) seems appropriate.

There appears to be only one other report of anucleate basidiospores among the Hymenomycetes. Basidiospores of *A. bisporus* are usually 4-nucleate (Raper & Raper, 1972) but may contain nil to eight nuclei (Lemke *et al.*, 1975), suggesting that all eight post-meiotic nuclei can migrate into one of the two spores on a basidium. As *P. noxius* has a high percentage of anucleate spores, the pattern of uneven distribution of nuclei among spores seems applicable to the fungus, and can be reconciled with the uneven development of spores on the one basidium. Two morphologically distinct states exist among interphase nuclei in fungal mycelia and spores: "resting" nuclei, and "migrating" nuclei. The former are spherical (or nearly so), and predominate in older mycelia, are much less frequent in young mycelia, and are the sole element in spores. Migrating nuclei assume shapes other than spherical (elongated to tear-shaped to attenuated); most nuclei in young mycelia are in this state, but this type is less frequent in older mycelia. Descriptions and locations of resting and migrating nuclei given for other fungal species (Shaw, 1953; Bakerspigel, 1957, 1958, 1959; Robinow, 1957; Ward & Ciurysek, 1961, 1962; Brushaber *et al.*, 1967; Griffin & Wilson, 1967; Knox-Davies, 1967) generally apply also to *P. noxius*.

As in *V. volvacea* (Chang & Ling, 1970), the direction of nuclear migration in *P. noxius* does not appear to be under the centriole control reported in *H. annosum* and several other species (Wilson & Aist, 1967). Nuclear migration was extensive throughout mycelia of *P. noxius* and nuclei were observed passing through dolipore septa and bridges between anastomosing hyphae. *Phellinus noxius*, therefore, fits the generalization of Raper and Flexer (1970) that dolipore septa only occur in those groups of fungi in which there is typically a rapid, extensive and oriented nuclear migration.

Nuclear division in fungal hyphae conforms to one of three general patterns, one mitotic and two amitotic. Essential features of mitotic division include progression through four distinct phases (prophase, metaphase, anaphase, telophase), the organization of contracted chromosomes on an equatorial plate at metaphase (which also facilitates counts of chromosomes), and the separation of daughter chromosomes (disjunction) at early anaphase (Knox-Davies & Dickson, 1960; Ward & Ciurysek, 1961, 1962; Hosford & Gries, 1966; Knox-Davies, 1967; Chang & Ling, 1970). In one of the amitotic patterns, nuclear chromatin first contracts, then elongates, constricts at the middle and finally separates to yield two daughter nuclei (Bakerspigel, 1957, 1959; Robinow, 1957; Chang & Ling, 1970). In the other, chromatids form double strands lying parallel to the long axis of the hypha; they then separate and finally assume a more rounded shape as daughter nuclei move in opposite directions along the hypha. Both mitotic and amitotic configurations have been recognized in the one fungus (Chang & Ling, 1970).

Division in *P. noxius*, together with such other Basidiomycetes as *H. annosum* (Brushaber *et al.*, 1967; Griffin & Wilson, 1967), *P. weirii* (Hansen, 1979a) and *Puccinia* spp. (Harder, 1976; Wright & Lennard, 1978), fits the second pattern. Generally, the nucleolar displacement and centriole replication found in *P. noxius* does not feature in descriptions of the pattern, but have been recorded, the former in *Puccinia* (Harder, 1976; Wright & Lennard, 1978), and the latter in *Entophlyctis* sp. (Powell, 1975).

During amitosis, the entire nucleus divides or fragments directly into two portions; and there is no evidence (as there is in mitosis) of equal chromosomal or even chromatin distribution to daughter nuclei (Ward & Ciurysek, 1962). In the present studies, cytological observations

revealed that (i) many nuclei dividing amitotically yielded daughter nuclei of noticeably unequal chromatin, and presumably chromosomal content; (ii) volumes of resting nuclei varied widely, even within the one cell; and (iii) resting nuclei in an unpigmented variant were larger than those in its pigmented counterpart. Nuclear size has been shown to be a useful indicator of ploidy in fungal isolates (Section 2.7.2) and thus polyploidy is indicated in *P. noxius*.

6.4 EXPERIMENT 3: POLYPLOIDY IN SPORES AND VEGETATIVE MYCELIA

6.4.1 Introduction

Wide variation was observed in the size of haemalum-stained nuclei within arthrospores, and within individual cells of field isolates and monosporous mycelia (Section 6.3). Also, the chromatin content of Giemsa-stained nuclei appeared to vary, even between daughter nuclei of the one parent nucleus.

These observations suggest the possible presence of different levels of ploidy among nuclei in spores and vegetative mycelia of the fungus. However, this could not be confirmed by chromosomal counts as nuclear division was not according to classical mitotic configurations and nuclear organelles which could be identified unequivocally as chromosomes were not seen.

In the following studies, evidence for polyploidy in the fungus was sought using three different methods: (1) examination of the correlations between nuclear number and spore volume, and nuclear volume per spore and spore volume; (2) Feulgen-DNA microspectrophotometry; and (3) determinations of nuclear volumes. Material studied comprised mycelia of field isolates, asexual and sexual spores and monosporous mycelia.

6.4.2 Materials and Methods

The isolates studied were those used in the experiment reported in Section 6.1. One of the basidiocarps provided the basidiospores and an isolate from its context, the arthrospores.

Feulgen-DNA microspectrophotometry was performed on nuclei in cultures of the three pigmented isolates (2250, LB3 and LB5S). To prepare the Feulgen reagent, 0.5 g basic fuchsin was added to 100 ml distilled water and dissolved by heating to boiling point. The solution was then cooled to 52-54^{o}C and filtered through 42 Whatman's paper. Potassium metabisulphite (0.5 g) was dissolved in 10 ml 1N HCl and added to the filtrate when it had cooled to 25^{o}C. The mixture was left overnight in a tightly stoppered bottle wrapped in aluminium foil. To decolorize the solution (the reagent should be a pale straw colour to colourless), approximately 0.2 g activated charcoal was added for 30 min and then filtered off. The reagent was stored in a refrigerator in an airtight bottle wrapped in aluminium foil.

A culture of each isolate was grown on cellophane overlaying malt-extract agar using the method described earlier (Section 6.2.2). Three strips of cellophane bearing hyphae of the advancing margin were cut from each culture and placed in a 6:1:1 mixture of absolute ethanol, glacial acetic acid and lactic acid for 10 min, immersed in 95% ethanol for 5 min, and then in 70% ethanol for 10 min. They were then washed in distilled water for 10 min, hydrolysed in 5N HCl at room temperature for 40-45 min, washed again in distilled water for 5 min, and stained with the Feulgen reagent for 4 hr. The cellophane and cytoplasm were cleared in two changes (each of 5 min) of SO_2-water (5 ml of 10% $K_2S_2O_5$; 5 ml 1N HCl; and 90 ml distilled water). The material was washed for 20 min

in distilled water, dehydrated through a graded series of absolute ethanol and xylene, to pure xylene, mounted in Eukitt (O. Kindler, Freiburg, West Germany) on microscope slides and covered with glass slips.

Measurements of DNA content of individual Feulgen-stained nuclei were performed on a Zeiss 05 photometer system at 570 nm; integrated absorbance of specimens was automatically calculated by running the APAMOS Modified program on a Digital PDPI2 computer interfaced to the photometer (Gould, 1979). Data were obtained for 100 spherical nuclei in apical cells in the advancing margin on each strip; the sample for each isolate therefore comprised 300 nuclei.

As discussed in Section 2.7.1, the intensity of nuclear staining with the Feulgen reagent varied widely among staining sessions and material satisfactory for microspectrophotometry was obtained in only one session. Measurements of ploidy level for the remainder of the experiment were therefore based on determinations of volumes of spherical nuclei stained with Meyer's haemalum; these data were obtained from material prepared for Experiment 1 of this series. Measurements of nuclear diameters (and of spores, where appropriate) were made at 2000X magnification with a Wild M20 microscope using bright field optics and a red filter. An eyepiece scale fitted to the microscope was calibrated with a stage micrometer; accuracy of measurement was ±0.125 μm.

6.4.3 Results

6.4.3.1 The Evidence for Polyploidy

Correlations between Number and Volume of Nuclei in Spores, and Spore Volume. Thirty arthrospores were selected at random from a culture of each of two isolates. The number of nuclei and total nuclear volume were determined for each spore, together with spore volume. The

coefficients of the correlation between number of nuclei and spore volume, and nuclear volume and spore volume were calculated for each isolate.

If nuclei were all of the same ploidy, it would be expected that the number of nuclei would be closely correlated with spore volume. However, the results (Table 6.10) clearly show that there is virtually no correlation between the number of nuclei and spore volume; on the other hand, a good correlation exists between nuclear volume and spore volume.

Table 6.10 Correlations between number of nuclei and arthrospore volume and total nuclear volume and arthrospore volume in two isolates of *P. noxius*

Isolate	Parameter correlated with spore volume	Correlation coefficient
LB3	Number of nuclei	-0.16
	Volume of nuclei	0.75
LB3U	Number of nuclei	0.17
	Volume of nuclei	0.85

Hence, the ploidy of nuclei in arthrospores of *P noxius* appears to vary.

Microspectrophotometry. Five modal peaks of DNA content were found among nuclei of the three pigmented isolates (Fig. 6.7). Distinct peaks in the frequencies of nuclei with varying DNA contents occur at regular intervals of $\log_{10}$ absorbance of 0.3, in isolates LB3 and LB5S; these peaks are absent in isolate 2250 at lower DNA contents. Every 0.3 increase in $\log_{10}$ absorbance represents a doubling of DNA content. Contents of DNA in nuclei vary from 1C (the modal peak of lowest DNA content) to 16C with 4C being the most frequent value. The presence of nuclei with DNA contents intermediate between peaks may indicate the occurrence of aneuploids.

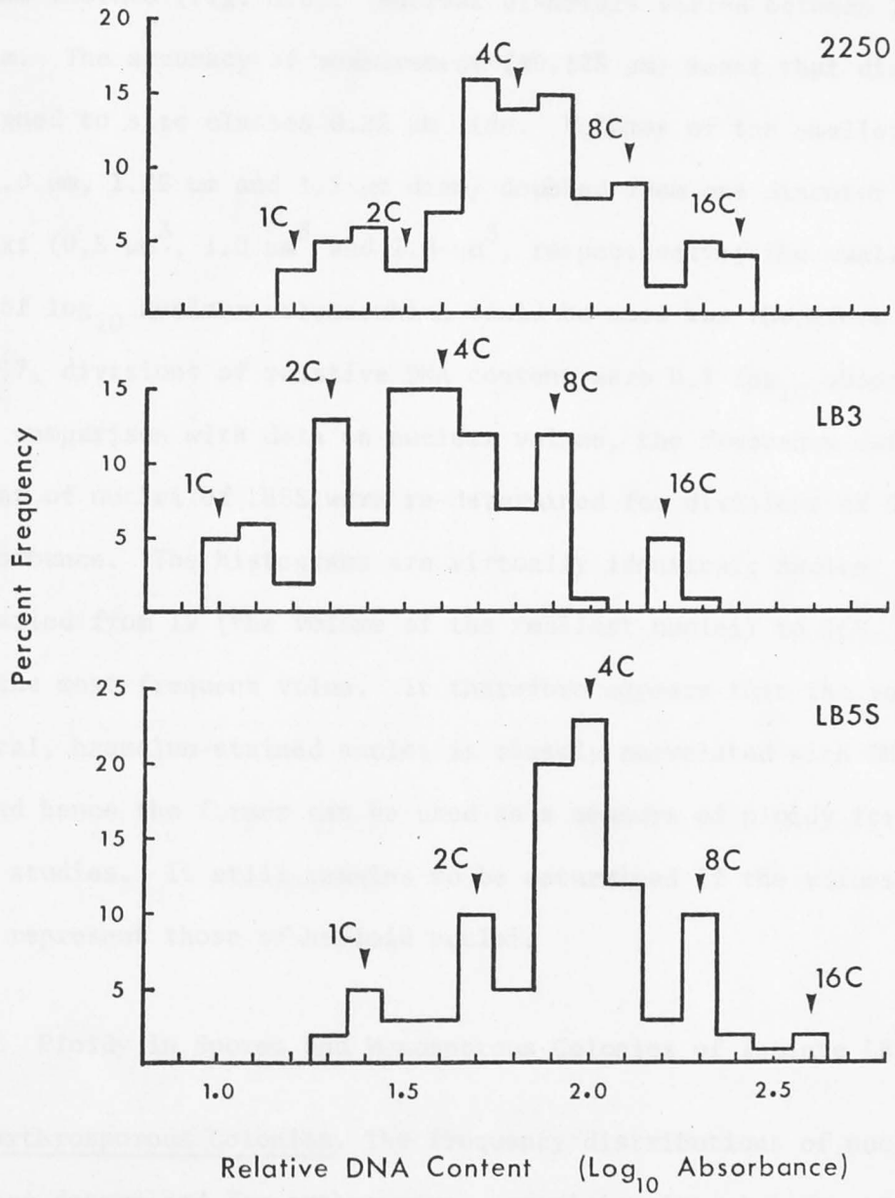


Figure 6.7 Relative DNA content of nuclei in apical cells of pigmented isolates 2250, LB3, and LB5S

Nuclear Volume. The frequency distribution of 300 spherical haemalum-stained nuclei of LB5S were compared with that of the DNA content of nuclei of the same isolate (Fig. 6.8). Nuclear diameters varied between 1.0 µm and 2.5 µm. The accuracy of measurement (±0.125 µm) meant that diameters were assigned to size classes 0.25 µm wide. Volumes of the smaller nuclei (1.0 µm, 1.25 µm and 1.5 µm diam) doubled from one diameter class to the next (0.5 μm^3, 1.0 μm^3 and 1.8 μm^3, respectively); the smallest division of $\log_{10}$ nuclear volume which could be used was therefore 0.3. In Fig. 6.7, divisions of relative DNA content were 0.1 $\log_{10}$ absorbance; hence for comparison with data on nuclear volume, the frequency data for DNA content of nuclei of LB5S were re-determined for divisions of 0.3 $\log_{10}$ absorbance. The histograms are virtually identical; nuclear volumes varied from 1V (the volume of the smallest nuclei) to 16V, with 4V being the most frequent value. It therefore appears that the volume of spherical, haemalum-stained nuclei is closely correlated with DNA content and hence the former can be used as a measure of ploidy for the remaining studies. It still remains to be determined if the values of 1C and 1V represent those of haploid nuclei.

6.4.3.2 Ploidy in Spores and Monosporous Colonies of Isolate LB5S

Monoarthrosporous Colonies. The frequency distributions of nuclear volumes were determined for arthrospores comprising inoculum for monosporous colonies, apical cells of monosporous colonies after 24 hr incubation, and arthrospores of monosporous colonies incubated for 96 hr, and compared with that for apical cells in the parent isolate. The five levels of nuclear volume (1V, 2V, 4V, 8V, 16V) found in the parent were present at all stages in the development of monosporous colonies, and

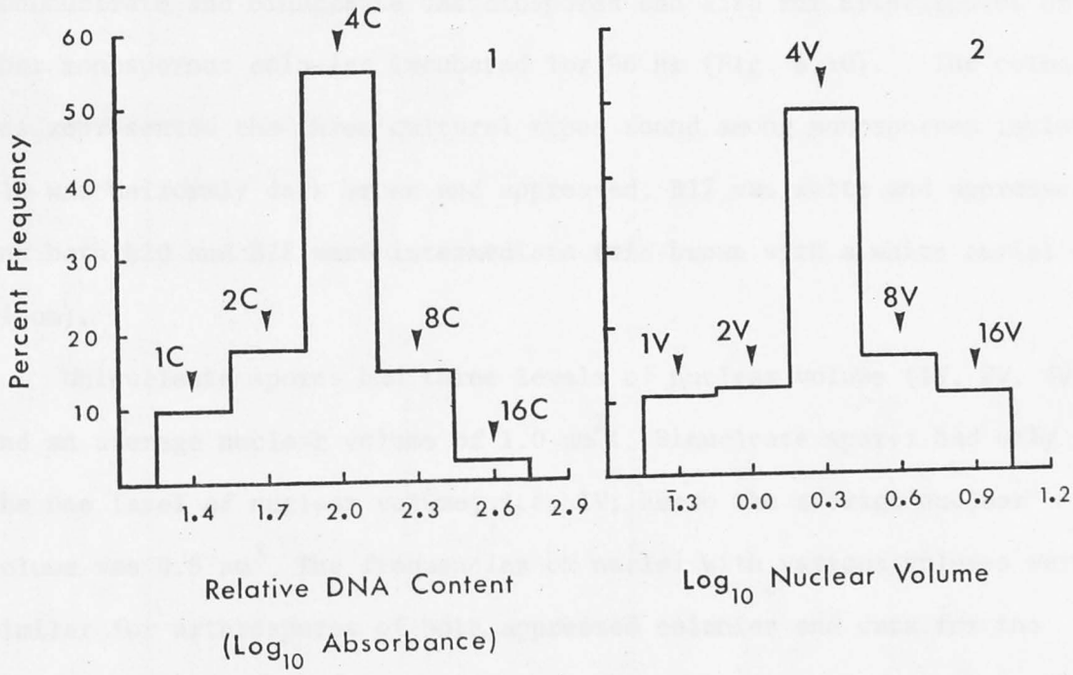


Figure 6.8 Relative DNA content and volumes of nuclei in apical cells of the pigmented isolate LB5S
1 - DNA content; and 2 - Nuclear volumes

the most frequent value of nuclear volume was 4V (Fig. 6.9). Average nuclear volumes (2.6-2.7 μm^3) in the parent isolate, arthrospore inoculum, and arthrospores of colonies incubated for 96 hr did not differ significantly, while that in apical cells of young colonies was significantly less (1.9 μm^3).

Monobasidiosporous Colonies. Nuclear volumes were determined for mononucleate and binucleate basidiospores and also for arthrospores of four monosporous colonies incubated for 96 hr (Fig. 6.10). The colonies represented the three cultural types found among monosporous isolates: B12 was uniformly dark brown and appressed; B17 was white and appressed and both B20 and B28 were intermediate (mid-brown with a white aerial bloom).

Uninucleate spores had three levels of nuclear volume (1V, 2V, 4V) and an average nuclear volume of 1.0 μm^3. Binucleate spores had only the one level of nuclear volume, i.e. 1V; hence the average nuclear volume was 0.5 μm^3. The frequencies of nuclei with various volumes were similar for arthrospores of both appressed colonies and data for the two were pooled; they had three levels of nuclear volume from 1V to 4V, with 2V being the most frequent, and the mean nuclear volume was 1.0 μm^3. The frequency distributions of both intermediate colonies were similar also; they had four levels of nuclear volume, with 4V being the most frequent, and had a mean nuclear volume of 2.0 μm^3. The mean volume of all nuclei was 1.5 μm^3, approximately half that of the parent isolate (see also Fig. 6.9), and the most frequent level of nuclear volume was 2V.

6.4.3.3 Ploidy in Arthrospores of the Pigmented Isolate LB3, and its Unpigmented Variant

Results are presented in Fig. 6.11. Nuclei in arthrospores of the unpigmented variant had an average volume double that of those of the

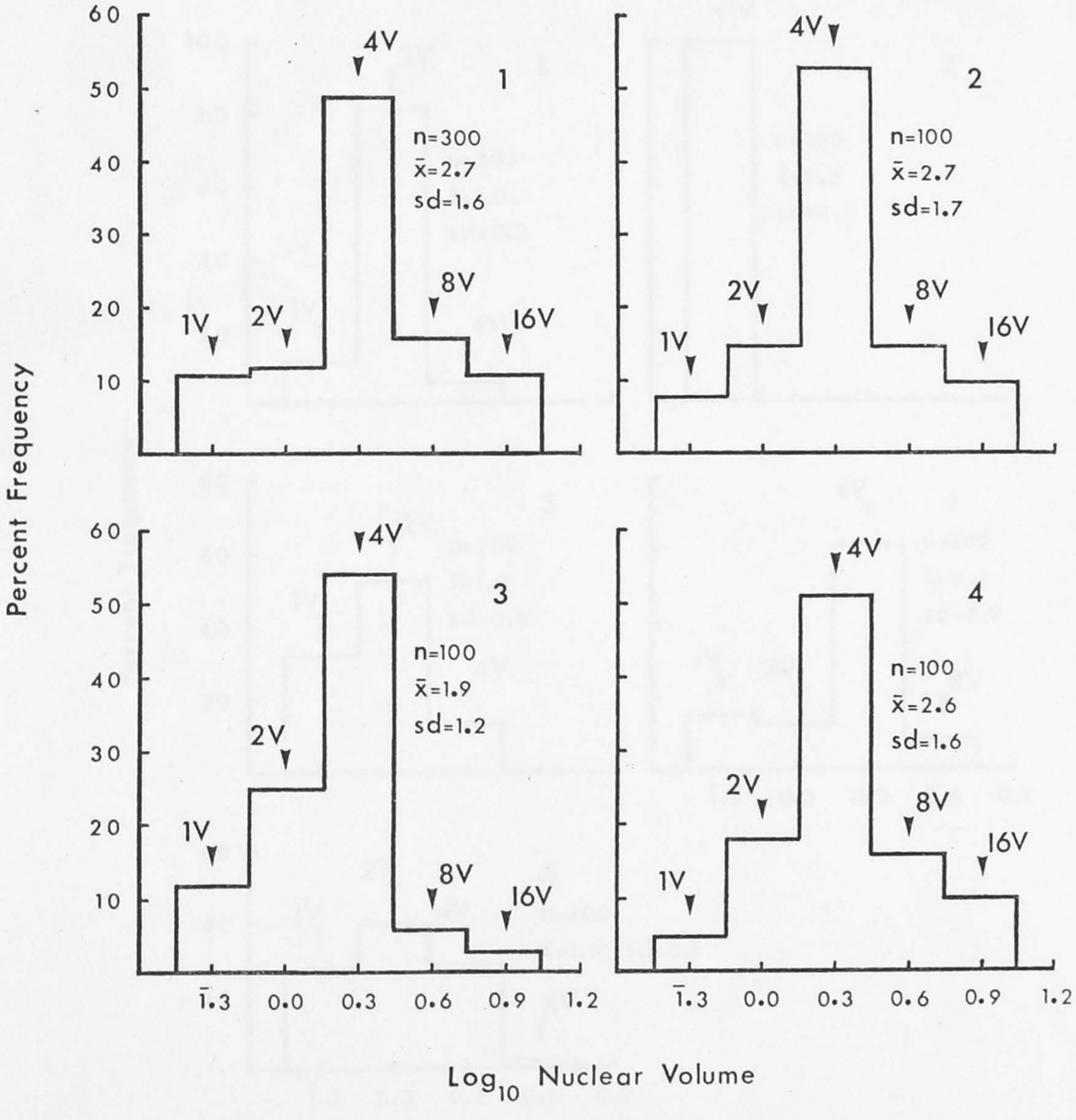


Figure 6.9 Nuclear volumes in cells at various stages of the development of monoarthrosporous colonies 1 - Apical cells of the parent isolate LB5S; 2 - Arthrospores comprising the inoculum for monosporous colonies; 3 - Apical cells of monosporous colonies after 24 hr incubation; and 4 - Arthrospores of monosporous colonies incubated for 96 hr

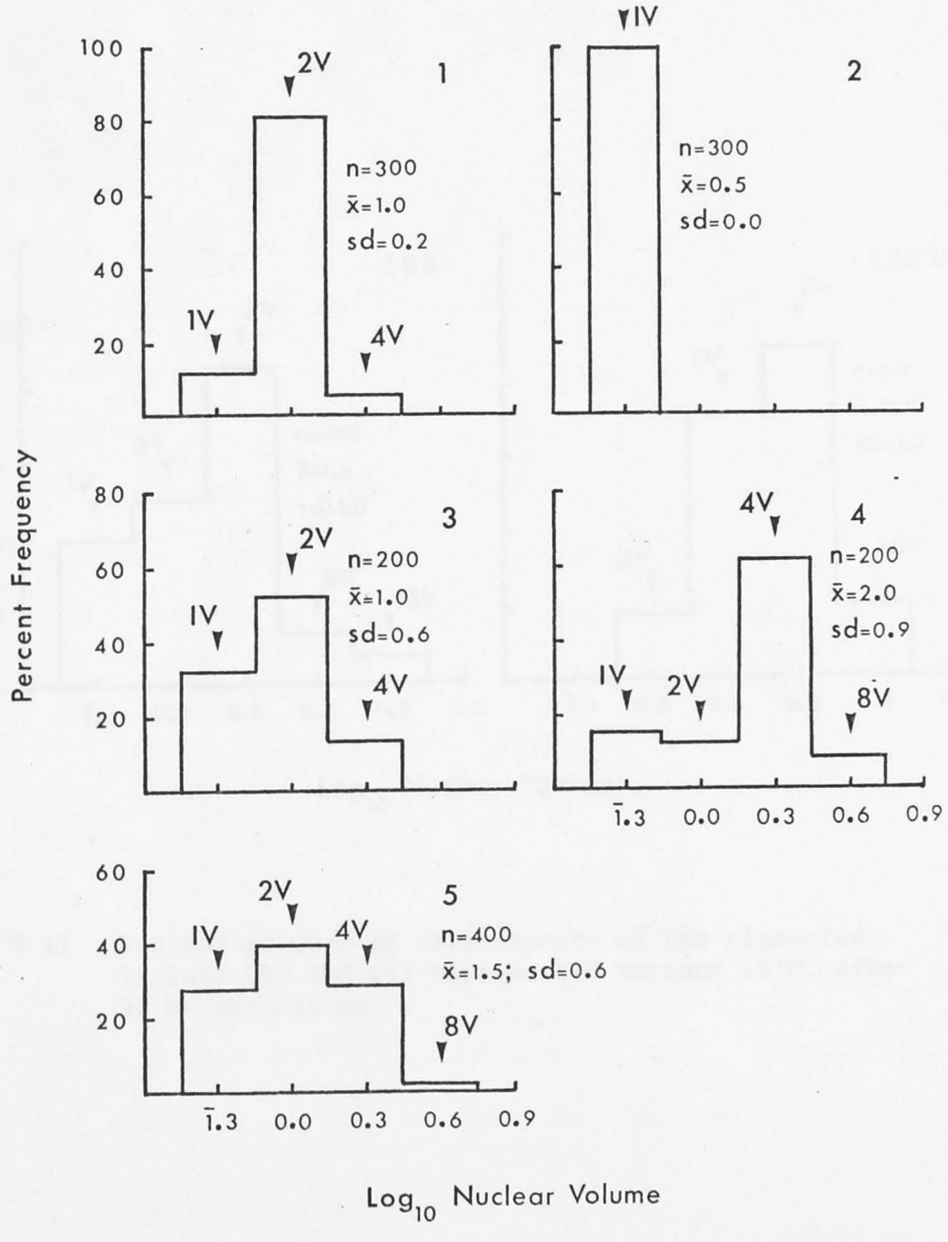


Figure 6.10 Nuclear volumes in basidiospores and in arthrospores of monosporous colonies after 96 hr incubation 1 - Uninucleate basidiospores; 2 - Binucleate basidiospores; 3 - Arthrospores of appressed monosporous colonies; 4 - Arthrospores of intermediate monosporous colonies and 5 - Arthrospores of all monosporous colonies

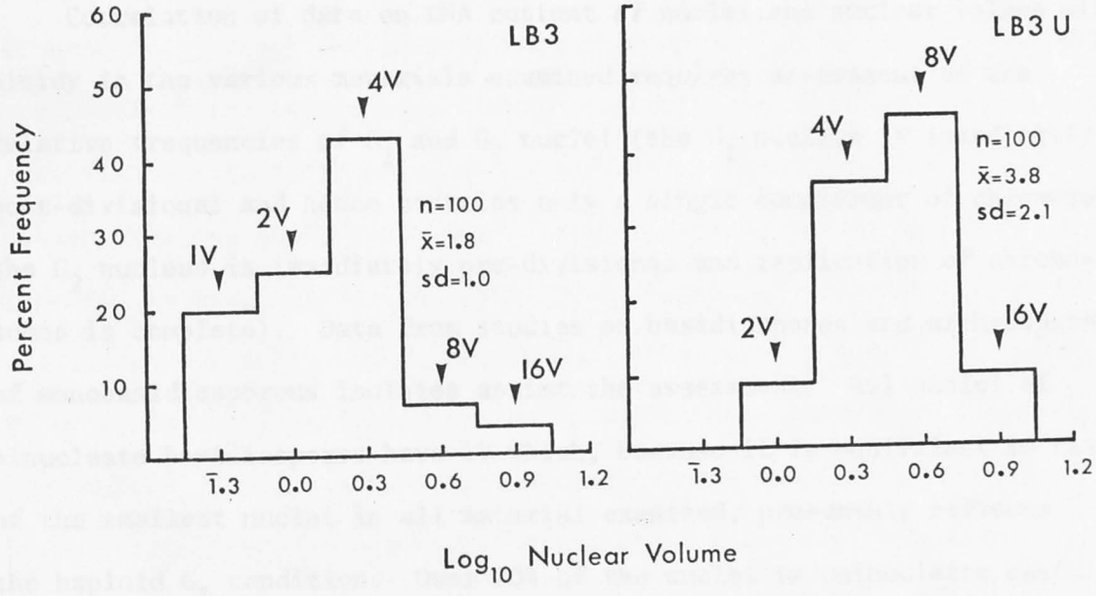


Figure 6.11 Nuclear volumes in arthrospores of the pigmented isolate LB3 and its unpigmented variant LB3U, after 96 hr incubation

pigmented isolate (3.8 μm^3 cf. 1.8 μm^3). Four levels of nuclear volume (2V to 16V) were present among the former, the most frequent being 8V; the latter had five levels (1V to 16V), with the most common value being 4V.

6.4.4 Discussion

Correlation of data on DNA content of nuclei and nuclear volume with ploidy in the various materials examined requires assessment of the relative frequencies of G_1 and G_2 nuclei (the G_1 nucleus is immediately post-divisional and hence contains only a single complement of chromosomes; the G_2 nucleus is immediately pre-divisional and replication of chromosomes is complete). Data from studies on basidiospores and arthrospores of monobasidiosporous isolates assist the assessment. All nuclei of binucleate basidiospores have 1V which, because it is equivalent to that of the smallest nuclei in all material examined, presumably reflects the haploid G_1 condition. Over 80% of the nuclei in uninucleate basidiospores have 2V volume; this value might represent that of nuclei in the replicated haploid (G_2) or diploid G_1 phase. Two patterns of distribution of nuclear volume in arthrospores are found among monobasidiosporous isolates: one has a peak at 2V, and the other, a peak at 4V. If nuclei of uninucleate basidiospores were haploid, only one pattern would be expected, and so they must be diploid G_1. A relationship between ploidy in basidiospores and nuclear volume in arthrospores of basidiospore isolates then emerges: basidiospores with haploid nuclei presumably yield isolates with nuclei of arthrospores mostly in the 2V condition, and those with diploid nuclei give rise to isolates where 4V nuclei predominate in their arthrospores. Arthrospores of isolates presumed to originate from basidiospores with haploid nuclei show a peak at 2V and a major

distribution toward the 1V value. This is suggestive of a lesser haploid G_1 phase and a major haploid G_2 phase. Similarly, G_2 diploid nuclei appear to predominate in arthrospores of the other isolates.

As the nature of post-meiotic events in basidia of *P. noxius* is unknown, the origin of diploid basidiospores is uncertain. However, there are two possibilities: the first is by non-disjunction of chromosomes during meiosis; and the second is by fusion of the two nuclei in binucleate spores. The former appears to operate in *Armillariella mellea* where polyploidy exists at all stages of the life cycle, but more specifically, in gill trama, the binucleate subhymenium, among post-fusion zygotes, among prophase II meiotic nuclei, and in basidiospores (Peabody *et al.*, 1978); the polyploid condition of prophase II nuclei was thought to arise through an incomplete meiotic process. In contrast to *P. noxius*, all basidiospores of *A. mellea* are uninucleate (Tommerup & Broadbent, 1974). Apparently, nuclear fusion has not been observed in basidiospores of other Hymenomycetes, but then neither has the high frequency of anucleate spores that is found in *P. noxius*. If, as in other Holobasidiomycetidae, four post-meiotic nuclei are produced and all migrate to spores, the mean value of nuclei per spore of *P. noxius* would be 1.0, considerably higher than the 0.7 recorded. It therefore appears that the uninucleate diploid condition may result from nuclear fusion in spores which received two haploid nuclei from the basidium. Some 13% of uninucleate basidiospores appear to be haploid. If the remainder are assumed to have been binucleate initially, the mean value of nuclei per spore would be 1.15. Some of the assumed diploids may be replicating haploids (since there is evidence of the presence of replicating diploids). If so, the mean value would be closer to 1.0. The lack of fusion in some binucleate spores appears to resemble a

mechanism in *Schizophyllum commune* in which the dominant allele *dik*$^+$ regulates dikaryosis (Koltin & Raper, 1968). The foregoing highlights the importance of developing a successful procedure for staining nuclei in basidia and attached basidiospores of *P. noxius*. An analysis of meiosis and post-meiotic events would help to resolve the equivocacy of the origin of diploid basidiospores.

The distributions of nuclear volumes in the tissue isolate LB5S, and the stages examined in the life cycle of its arthrospores, all extend from 1V to 16V, and peak at 4V. It is reasonable to assume that the nuclei in arthrospores of tissue and monoarthrosporous isolates would be in the same predominantly G_2 interphase condition as those of monobasidiosporous isolates. On the basis of the data presented here, it appears that the basic life cycle of *P. noxius* is diploid, and a heterogeneity in ploidy levels from haploid to octaploid is indicated. Arthrospores perpetuate this condition.

An increasing body of literature suggests that diploidy and polyploidy are more common in fungi than previously suspected (see the review of Rogers, 1973). In the Holobasidiomycetidae, documentation of these conditions is fragmentary, but they have been reported in *A. mellea* (Korhonen & Hintikka, 1974; Tommerup & Broadbent, 1975), *Coprinus lagopus* Fr. (Casselton, 1965) and other *Coprinus* spp. (Lu & Raju, 1970), *Heterobasidion annosum* (Ahrberg, 1975), and *S. commune* (Parag & Nachman, 1966; Koltin & Raper, 1968; Mills & Ellingboe, 1969). Diploidy and polyploidy in the somatic phase presumably arise through nuclear fusion, as in the parasexual cycle (Pontecorvo, 1956; Tinline & MacNeill, 1969; Webster, 1974), or by endomitosis.

Evidence for fusion has been largely genetic (Pontecorvo, 1956; Burnett, 1975), but it has been observed cytologically with phase-

microscopy in living hyphae, e.g. in *A. mellea* and *S. commune* (Parag, 1968; Korhonen & Hintikka, 1974). Fusions in the latter species apparently were rare. They were more frequent in *A. mellea*, occurring in 3-8% of apical cells of dikaryons isolated from young gills of fresh basidiocarps and in dikaryons formed in compatible matings of single-spore isolates. Direct evidence of nuclear fusion was not found in the present cytological studies on *P. noxius*, but it probably occurs. The multinucleate condition of hyphae would presumably afford a greater opportunity for fusion than the dikaryotic condition of the above two species. Available evidence from other studies suggest that nuclear fusion is unlikely to account for all polyploid nuclei in *P. noxius*, hence endomitosis is likely also.

As a consequence of non-disjunction in mitosis, a proportion of the divisions of diploid or polyploid nuclei yields aneuploids; aneuploids are unstable, but can divide repeatedly, progressively losing chromosomes until euploidy is regained. The evidence for the existence of aneuploids, as with nuclear fusion, comes mostly from genetical analyses. However, chromosomal counts in cytological studies have confirmed their presence in fungi (Emmerson & Wilson, 1954; Knox-Davies & Dickson, 1960; Knox-Davies, 1967; Brody & Williams, 1974; Olson & Borkhardt, 1978). Microspectrophotometrical analyses showed that there are nuclei in mycelia of *P. noxius* with DNA contents intermediate between those of euploids. These nuclei may be in the S-phase of replication, or aneuploids, or both (in the S-phase, which occurs between the G_1 and G_2 phases, the nucleus is replicating its chromosomes). Data from these studies do not allow calculation of the relative proportions. The cytological examinations reported herein suggest the existence of aneuploids in the hyphae of *P. noxius* (Section 6.3.3.2).

An interesting variation exists in the pattern of nuclear-DNA distribution in isolate 2250: whereas euploid peaks are prominent for isolates LB3 and LB5S, absorbance values for euploids in 2250 are equivocal. Either 1.2 or 1.4 $\log_{10}$ absorbance could have been selected to represent the 1C value, and peaks are less pronounced than in LB3 or LB5S. This suggests that aneuploidy may be more frequent in this isolate than the other two.

The mechanism for the stable tetraploid in the unpigmented variant of *P. noxius* is unknown. Three possible explanations of this unusual event are (1) fusion of diploid nuclei, (2) endomitosis, and (3) a spontaneous doubling of chromosome number in diploid nuclei. Earlier in this discussion, (1) and (2) were offered as explanations for heterogeneous polyploidy in somatic hyphae of *P. noxius*; in either case, tetraploids and octaploids appear to be unstable and reduce to the predominant diploid condition, presumably via aneuploidy. The case for (3) is strengthened by the absence of haploid nuclei (G_1) in the variant.

Many authors have shown fluorescence photometry or microspectrophotometry to be useful in studying ploidy in fungi (Bryant & Howard, 1969; Lemke *et al.*, 1975; Williams & Mendgen, 1975; Haskins, 1977; Peabody *et al.*, 1978); the present studies confirm the usefulness of the method. Considerable problems were encountered, however, with the Feulgen-staining method; the Schiff's reagent used was basic fuchsin (pararosaniline). Reagents which have been used with success in other studies include acridine yellow, acriflavine, acrinol, auramin-O, bisbenzimide H33258, and coriphosphine (Bohm & Sprenger, 1968; Lemke *et al.*, 1975; Forche & Blaschke, 1978). These should be tested for efficacy in future studies of this nature on *P. noxius*.

6.5 CONCLUSION

Mycelia of tissue isolates of *P. noxius* comprise cells which are multinucleate, and presumably heterokaryotic. Data from the present studies also suggest that the fungus has a somatic phase which is predominantly diploid, but has a degree of heterogeneity in ploidy levels. Diploid and polyploid nuclei may be homozygous (through endomitosis) or heterozygous (through fusion of genetically dissimilar nuclei). The parasexual cycle described by Pontecorvo and Roper (1952) explains variation in the somatic phase of fungi. The cycle comprises the sequence:- fusion of dissimilar nuclei: mitotic recombination and: haploidization. Haploidization occurs by successive mitoses from a hyperdiploid nucleus derived from accidental non-disjunction of the diploid nucleus, probably by the loss of chromosomes. There appears to be no reason why diploidization of supradiploids (i.e. tetraploids and octaploids) should not proceed by a similar mechanism. A model (Fig. 6.12) is then proposed to explain variation in the somatic phase of *P. noxius*. This model is based on nuclear mechanisms which appear to operate in the fungus.

Ploidy in *P. noxius* appears to be in a state of flux. Proposed major pathways lead to the diploid condition through fusion or endomitosis of haploid nuclei, and through chromosomal losses in divisions following non-disjunction of supra-diploid nuclei. Minor pathways give rise to the haploid condition through non-disjunction and chromosomal losses, and to the supradiploid condition through nuclear fusion or endomitosis. Tetraploid variants may arise through nuclear fusion, endomitosis, or a spontaneous doubling of chromosomes; the latter is the most likely mechanism as these variants apparently lack haploid nuclei. The mechanism responsible for the stable, predominantly tetraploid condition is probably similar to that proposed for the diploid condition.

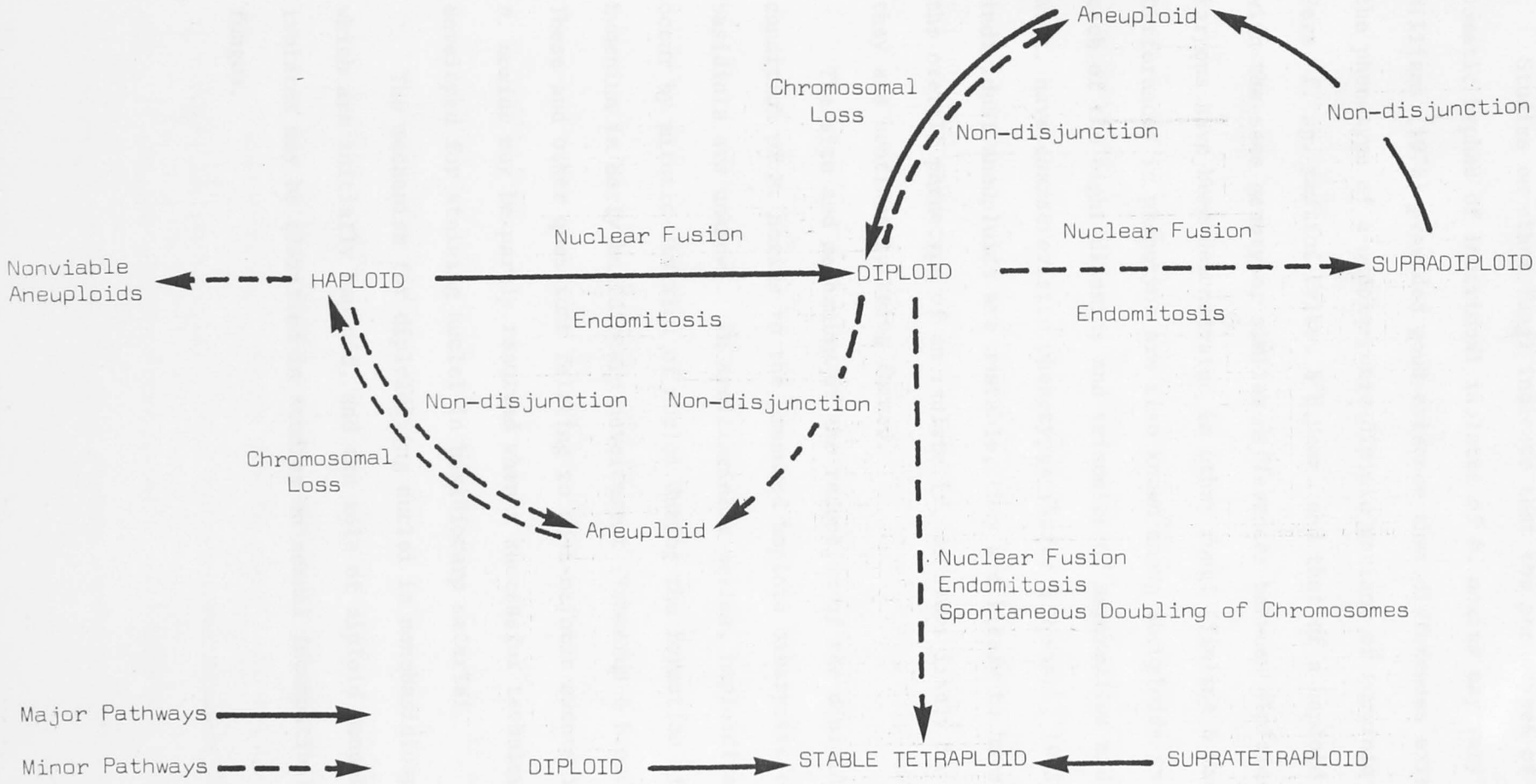


Figure 6.12 Proposed model for nuclear mechanisms which appear to operate in somatic hyphae

Studies on other fungi indicate that the phenotypes of nuclei in somatic hyphae of individual isolates of *P. noxius* may vary widely. Williams (1975) provided good evidence that differences exist between the phenotype of a monokaryotic diploid variant of *Puccinia graminis* Pers. f. sp. *tritici* Eriks. & E.Henn. and that of a haploid dikaryon with the same genotype; similar differences between diploids and heterokaryons have been demonstrated in other fungi (Tinline & MacNeill, 1969). Differences in phenotype are also known among aneuploids in the fungi; each of the eight disomics and trisomics of *Aspergillus nidulans* (Eidam) Wint. have characteristic phenotypes (Kafer & Upshall, 1973). Although individual aneuploids are unstable, they are likely to have some effect on the overall phenotype of an isolate if, as seems likely in *P. noxius*, they are continually being formed.

The site and mechanism of the reduction of the diploid somatic condition of *P. noxius* to the presumed haploid dikaryotic condition in basidiola are unknown. In *Armillariella mellea*, haploidization appears to occur by mitotic division of nuclei during the formation of the subhymenium in early basidiocarp development (Tommerup & Broadbent, 1975). These and other questions relating to post-meiotic events in basidia of *P. noxius* may be partly resolved when a successful technique has been developed for staining nuclei in basidiocarp material.

The mechanism for diploidizing nuclei in monobasidiosporous isolates which are initially haploid, and the role of diploid monobasidiosporous isolates may be clarified in studies on sexual incompatibility in the fungus.

CHAPTER 7

SEXUAL AND VEGETATIVE INCOMPATIBILITY

7.1 INTRODUCTION

Neither sexual nor vegetative incompatibility has been examined previously in *P. noxius*. Sexuality has been investigated in a number of other species in the genus. Only one, *P. gilvus*, was determined to be homothallic, as single basidiospore isolates regularly formed basidiocarps (Hirt, 1928). *Phellinus tremulae* is described as bipolar (Niemelä, 1974) but the author did not specify how this was determined. *Phellinus igniarius* and *P. weirii* are reported as tetrapolar (Nobles, 1948; Hansen, 1979b), but apparently there is some doubt about the record for the former species (see Hansen, 1979b). Other species (e.g., *P. nigricans, P. populicola*) are heterothallic but the system of incompatibility was not determined (Niemelä, 1975). Thus, sexual incompatibility in the genus, and in the family (Hymenochaetaceae) generally, is poorly understood.

Part of the difficulty in determining the system of incompatibility in the Hymenochaetaceae is probably due to the absence of clamp connections on somatic mycelia (Hirt, 1928; Nobles, 1965; Niemelä, 1972, 1974, 1975, 1977b; Hansen 1979b). Thus incompatibility cannot be established in the traditional way. Hansen (1979b) recognized compatible pairings of monobasidiospore isolates of *P. weirii* using a number of characters, viz. (i) the production of basidiocarps, (ii) dramatic changes in the appearance of cultures and (iii) changes in the nuclear condition of

mycelia (heterokaryotic mycelia are irregularly binucleate, whereas homokaryotic mycelia are multinucleate (Hansen, 1979a)). As discussed previously (Section 6.2.4), none of these characters appear useful for studies with *P. noxius*.

Vegetative incompatibility has been investigated in many Hymenomycetes. Some of these species do not form clamp connections on cultured somatic mycelia, for example, *Armillariella mellea* (Adams, 1974; Shaw & Roth, 1976), *P. weirii* (Hansen, 1979b), and *Polyporus schweinitzii* Fr. (Barrett & Uscuplic, 1971). These species, like many other Hymenomycetes (see Section 2.8.2), form lines of demarcation in culture when isolates of genetically distinct origin are paired. Such lines are not formed between isolates of the same origin.

The following studies were therefore concerned with variation in *P. noxius* that may be due to heterokaryosis, another nuclear mechanism known to generate genetic diversity in fungi (Raper, 1966; Tinline & MacNeill, 1969; Webster, 1974; Burnett, 1976). Heterokaryosis (the occurrence of two or more genetically different nuclei in the one mycelium) may arise through mutation of a nucleus in a homokaryotic mycelium, or through anastomoses between genetically different mycelia. Only the latter origin of heterokaryosis was considered. The system of sexual incompatibility, interfertility, and vegetative incompatibility were investigated in these studies.

7.2 MATERIALS AND METHODS

The method of isolate preparation followed that given in Section 2.1. Field isolates were derived from two hosts and a range of geographical locations, and were those used in previous experiments (Section 4.2.1). Monobasidiospore isolates came from only the one basidiocarp and included

representatives of the three cultural types found among the isolates (Section 3.2.2.3). Monoarthrospore isolates were derived from cultures of a context isolate from the basidiocarp which provided the basidiospores, and were freshly prepared for each occasion they were used.

The system of sexual incompatibility was investigated by pairing monobasidiospore isolates in all possible combinations.

Interfertility was examined with the aid of the Buller phenomenon (Section 2.8.1) by pairing monobasidiospore isolates with monoarthrospore isolates, and with field isolates.

Vegetative incompatibility was studied by pairing monoarthrospore isolates in all possible combinations, by pairing monoarthrospore isolates with field isolates, and by pairing field isolates in all possible combinations.

Inoculum cultures were prepared by growing each isolate for 4 days from a central inoculation in petri dishes containing malt-extract agar (MEA: 10.0 g Difco Bacto malt-extract; 20.0 g Difco Bacto-Agar; 1000 ml distilled water). Discs, 4 mm diameter, were cut from the advancing margin of inoculum cultures and were paired 2 cm apart in 90 mm diameter petri dishes containing 30 ml MEA. Dishes were then incubated at 25°C in the dark. They were examined weekly for 6 weeks and interactions between paired mycelia were noted. Photographic records of various interactions were with an Asahi Pentax KM camera using Kodak Panatomic X film.

7.3 RESULTS

System of Sexual Incompatibility. Pairings of monobasidiospore mycelia failed to produce basidiocarps or obvious changes in cultural appearance.

Six different interactions were recognized in pairings comprising

mycelia derived from different basidiospores, viz. (i) isolates intermingled freely with no obvious interaction (designated the code "I"); (ii) a "barrage" developed consisting of mounded mycelia and dark pigmentation of the agar (MB); (iii) mycelia grew side by side and did not overlap, and the agar was darkly pigmented under the line of confrontation (NP); (iv) as for (iii) but the agar was not pigmented (NU); (v) mycelia overlapped in a zone about 5 mm wide, and the agar was darkly pigmented under overlapping mycelia (OP); and (vi) as for (v) but the agar was not pigmented (OU). A seventh type of interaction was recorded in one of the pairings of mycelia derived from the same basidiospore, (vii) a "barrage" consisting of sparse mycelia, indicating mutual aversion (A). Various interactions are illustrated in Fig. 7.1.

Conventional terminology for interactions between monobasidiospore mycelia (viz., compatible, hemicompatible, and noncompatible - see Raper, 1966) does not appear appropriate for those recorded in the present studies. If compatible interactions occurred, they could not be separated morphologically from other interactions. Hence, the terms "interactive" and "non-interactive" are used; the former is employed in instances where distinct interactions were recorded; the latter where paired mycelia intermingled freely.

Monobasidiospore isolates are almost certainly not homothallic because, as demonstrated in previous experiments (Chapters 3-6), they usually differed in cultural appearance from a context isolate prepared from the same basidiocarp and in certain physiological traits, in their pathogenicity on lupin, and in nuclear condition.

Interactions could not be arranged in mating tables conforming to either bipolar or tetrapolar patterns, and hence results are presented with the isolates in numerical order (Table 7.1).

Figure 7.1 Various interactions of monobasidiospore isolates from a single basidiocarp

1-3. Isolates intermingle with no obvious interaction ("I" in the text)

4. "Barrage" consisting of mounded mycelia along confrontation zone, and dark pigmentation of agar (MB)

5. Isolates grow side by side and do not overlap or intermingle, and there is no obvious interaction (NU)

6. As for NU, but agar is darkly pigmented under common margin (NP)

7. Isolates overlap, and agar is darkly pigmented under overlapping mycelia (OP)

8. "Barrage" consisting of sparse mycelia along zone of confrontation (A)

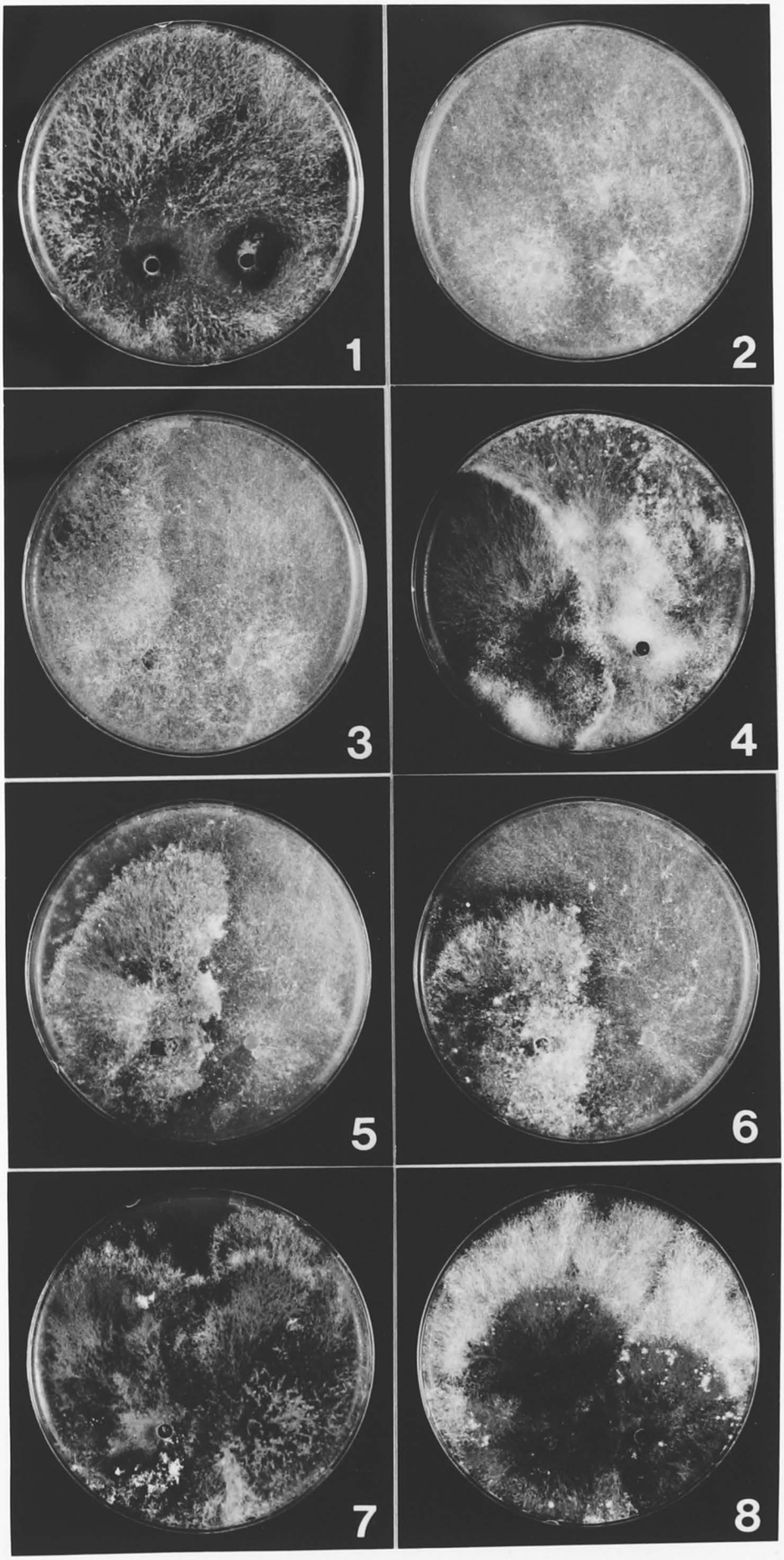

1
2
3
4
5
6
7
8

Table 7.1 Interactions of monobasidiospore isolates from a single basidiocarp paired in all combinations

Isolate Number	1	4	5	8	9	10	12	15	17	18	20	22	23	28	29
1	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I
4		I	I	NU	I	I	I	I	I	I	OU	I	I	MB	I
5			NU	I	I	I	NU	I	I	I	I	NU	OU	I	NU
8				A	I	NU	NU	NU	I	I	OU	NU	NU	NP	NP
9					I	I	MB	I	I	I	NU	I	I	I	I
10						I	I	I	I	I	I	I	I	I	I
12							NU	I	I	I	I	NP	I	MB	I
15								I	I	I	OP	I	I	MB	I
17									I	I	I	I	I	I	I
18										I	I	I	I	I	I
20											NU	NU	NU	NU	NU
22												I	I	I	OU
23													I	I	I
28														I	NU
29															I

I : Isolates intermingle with no obvious interaction

MB : "Barrage" consisting of mounded mycelia along confrontation zone, and dark pigmentation of agar

NU : Isolates grow side by side and do not overlap or intermingle, and there is no obvious interaction

NP : As for NU, but agar is darkly pigmented under common margin

OU : Isolates overlap about 5 mm, but there is no other obvious interaction

OP : As for OU, but agar is darkly pigmented under overlapping mycelia

A : "Barrage" consisting of sparse mycelia along zone of confrontation

The interactions of paired mycelia from the same basidiospore help to provide a possible interpretation of the apparently complex sexual incompatibility system operating in the fungus. Such pairings (i.e., A = in bipolar species, and A=B= in tetrapolar species) should be non-compatible, and are usually manifested in other Hymenomycetes by

morphologically characteristic interactions (Raper, 1966; Neuhauser & Gilbertson, 1971; Korhonen, 1978a). In the present studies, 11 such pairings were non-interactive. This suggests that each of these isolates might be at least partially self-compatible, and thus possibly heterokaryotic for mating type. Further evidence of the possible heterokaryotic nature of some basidiospore isolates comes from the pairing of isolate 8 with itself: The A-type interaction recorded resembles the A=B≠ or A≠B= interactions recorded elsewhere between homokaryotic mycelia (Raper, 1966; Korhonen, 1978a).

Only three isolates (5, 12, 18) gave interactions which suggested that each might be self-noncompatible, and thus homokaryotic for mating type. Pairings of these isolates with each other further indicate that the mating factors of isolates 5 and 12 were similar, while those of isolate 20 were different (Table 7.2). However, all three isolates differed in their interactions with other isolates, and therefore, appear to have different mating factors (Table 7.3). The results of these mating studies (Table 7.1) show that most isolates differed in their interactions with other isolates, and examples of three of these are included also in Table 7.3. Isolates which had similar interactions were 1, 17 and 18 (which freely intermingled with all isolates); and isolates 4 and 15.

Table 7.2 Interactions between three monobasidiospore isolates which appear to be self-incompatible

Isolate Number	5	12	20
5	NU	NU	I
12	NU	NU	I
20	I	I	NU

I : Isolates intermingle with no obvious interaction

NU: Isolates grow side by side and do not overlap, and the agar is not pigmented

Table 7.3 Interactions between six monobasidiospore isolates and other monobasidiospore isolates

Isolate Number	1	4	5	8	9	10	12	15	17	18	20	22	23	28	29
5	I	I	NU	I	I	I	NU	I	I	I	I	NU	OU	I	NU
12	I	I	NU	NU	B	I	NU	I	I	I	I	NP	I	B	I
20	I	OU	I	OU	NU	I	I	OP	I	I	NU	NU	NU	NU	NU
8	I	NU	I	A	I	NU	NU	NU	I	I	OU	NU	NU	NP	NU
1	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I
4	I	I	I	NU	I	I	I	I	I	I	OU	I	I	MB	I

I : Isolates intermingle with no obvious interaction

MB : "Barrage" consisting of mounded mycelia along confrontation zone, and dark pigmentation of agar

NU : Isolates grow side by side and do not overlap, and the agar is not pigmented under the line of confrontation

NP : As for NU, but agar is pigmented under common margin

OU : Isolates overlap about 5 mm, and the agar is not pigmented under overlapping mycelia

OP : As for OU, but agar is pigmented under overlapping mycelia

A : "Barrage" consisting of sparse mycelia along zone of confrontation

There was little correlation between interaction or non-interaction, and pigmentation of mycelia. All unpigmented isolates were non-interactive with each other, but not necessarily with all pigmented isolates; and most pairings of different pigmented mycelia were also non-interactive, but with some exceptions (Table 7.4). Similar results were obtained where isolates were paired with themselves (Table 7.5).

Table 7.4 Percent occurrence of various interactions of pigmented and unpigmented monobasidiospore isolates from a single basidiocarp (selfed isolates excluded)

Pairings	Interaction					
	I	MB	NU	NP	OU	OP
Unpig. x Unpig.	4%	-	-	-	-	-
Unpig. x Pig.	40%	3%	2%	-	-	-
Pig. x Pig.	30%	1%	12%	3%	4%	1%
All Pairings	74%	4%	14%	3%	4%	1%

I : Isolates intermingled
MB : Mounded "barrage"
NU : No intermingling; no pigmentation in confrontation zone
NP : No intermingling; but with pigmentation in confrontation zone
OU : Overlapping; no pigmentation
OP : Overlapping; with pigmentation

Table 7.5 Percentage occurrence of various interactions of selfed pigmented and unpigmented monobasidiospore isolates from the one basidiocarp

Pairings	Interactions						
	I	MB	NU	NP	OU	UP	A
Unpig. x Unpig.	20%	-	-	-	-	-	-
Pig. x Pig.	53%	-	20%	-	-	-	7%
All Pairings	73%	-	20%	-	-	-	7%

I : Isolates intermingled
MB : Mounded "barrage"
NU : No intermingling; no pigmentation in confrontation zone
NP : No intermingling; but with pigmentation in confrontation zone
OU : Overlapping; no pigmentation
OP : Overlapping; with pigmentation
A : Sparse "barrage"

Although the incompatibility system was not definitely established for *P. noxius*, there is evidence which suggests the species might be tetrapolar. Firstly, mycelia of most isolates appeared to be heterokaryotic for mating type; if the species were bipolar, these mycelia would contain nuclei of compatible mating types, and thus would be secondarily homothallic (i.e. A≠). However, as indicated previously, monobasidiospore isolates are almost certainly not homothallic. Secondly, in tetrapolar species 25% of pairings of siblings from the one basidiocarp are compatible, and 25% of pairings are noncompatible (the remainder are hemicompatible). In the present studies, 26% of pairings were interactive, and probably noncompatible (Table 7.4). Thus, the interactions recorded appear to agree better with a hypothesised tetrapolar incompatibility, but with numerous anomalies. It also appears that non-interactive pairings may be only hemicompatible, or that dikaryotization occurs only very slowly.

Interfertility. Basidiocarps or obvious changes in the appearance of cultures were not observed in these studies.

The interactions recorded between field and monobasidiospore isolates (Table 7.6) suggest that no one field isolate was potentially compatible with all monobasidiospore isolates, and thus, each can be at best only partially interfertile with the strain LB5S.

Field isolates varied in their patterns of interactions with the monobasidiospore isolates (Table 7.6). This suggests probable genetic differences among the field isolates. These results indicate that (i) isolates from the three geographical sources are genetically different; (ii) isolates from southeastern Queensland are genetically similar with respect to their interactions with monobasidiospore isolates; (iii) among

north Queensland isolates, those from the contexts of basidiocarps (2250, 2261, LB5S) are similar, but differ from the isolate from host tissue (LB5W); (iv) the Malaysian isolate LB2 is completely intersterile with strain LB5S, and thus, is genetically isolated from it; (v) the Malaysian isolates LB2 and LB3 are genetically different; (vi) there is a high degree of intersterility between isolate LB3 and strain LB5S and (vii) the unpigmented variant (LB3U) is genetically different to its pigmented counterpart, but its intersterility with the strain LB5S is less complete.

Table 7.6 Interactions of monobasidiospore isolates from the one basidiocarp (LB5S) and field isolates from various sources

Field Isolates	Basidiospore Isolates									
	1	4	8	9	10	12	17	20	28	29
1141C	I	I	MB	I	I	MB	I	I	MB	MBI
1354B	I	I	MB	I	I	MB	I	I	MB	MBI
1516	I	I	MB	I	I	MB	I	I	MB	MBI
2002	I	I	MB	I	I	MB	I	I	MB	MBI
2250	I	I	MB	I	MB	MB	I	I	I	I
2261	I	I	MB	I	MB	MB	I	I	I	I
LB5S	I	I	MB	I	MB	MB	I	I	I	I
LB5W	I	I	MB	I	MB	MB	I	I	MB	MBI
LB2	MB	MB	MB	MB	MB	MB	MB	MB	MB	MBI
LB3	I	I	MB	I	MB	MB	MB	MB	MB	MBI
LB3U	I	I	I	MB	I	MB	I	I	MB	I

I : Isolates intermingled
MB : Mounded "barrage" with light to moderate pigmentation in agar
MBI: Mounded "barrage" with intense pigmentation in agar
The first four field isolates come from S.E. Queensland; the second four come from N. Queensland and the last three are from Malaysia. LB3U is an unpigmented variant from isolate LB3

The interaction patterns of all monoarthrospore isolates with monobasidiospore isolates were similar (Table 7.7), suggesting that monoarthrospore isolates were genetically similar in this character. Only two of the monobasidiospore isolates (12, 28) were interactive with

arthrospore isolates, suggesting noncompatible interactions; however, these two isolates appear to differ genetically as the intensity of the agar pigmentation in the "barrage" zone was consistently different for the two isolates. The remaining basidiospore isolates were presumably either hemicompatible or compatible with the arthrospore isolates.

Table 7.7 Interactions of monobasidiospore and monoarthrospore isolates from the same basidiocarp

Arthrospore	Basidiospore Isolates									
Isolates	1	4	8	9	10	12	17	20	28	29
1	I	I	I	I	I	MBI	I	I	MBM	I
2	I	I	I	I	I	MBI	I	I	MBM	I
3	I	I	I	I	I	MBI	I	I	MBM	I
4	I	I	I	I	I	MBI	I	I	MBM	I
5	I	I	I	I	I	MBI	I	I	MBM	I

I : Isolates intermingled
MBI: Mounded "barrage" with intense pigmentation to agar
MBM: Mounded "barrage" with moderate pigmentation to agar

Vegetative Incompatibility

Monoarthrospore isolates intermingled freely with one another (Table 7.8) indicating, as would be expected, that they are genetically similar, that is, they are from the same "clone". They also freely intermingled with the field isolate from which they were derived (LB5S), but not with any other field isolate (Table 7.9). The latter results suggest that these other field isolates differ genetically from isolate LB5S. The consistency of the interactions among monoarthrospore isolates with any one field isolate (Table 7.9) again illustrates the genetic similarity of these isolates.

Table 7.8 Interactions of monoarthrospore isolates from the one field isolate (ex the context of basidiocarp collection LB5S)

Isolate Number	1	2	3	4	5	6	7	8	9	10
1	I	I	I	I	I	I	I	I	I	I
2		I	I	I	I	I	I	I	I	I
3			I	I	I	I	I	I	I	I
4				I	I	I	I	I	I	I
5					I	I	I	I	I	I
6						I	I	I	I	I
7							I	I	I	I
8								I	I	I
9									I	I
10										I

I : Isolates intermingle with no obvious interactions

Table 7.9 Interactions of monoarthrospore isolates from the one field isolate (ex the context of basidiocarp collection LB5S) and field isolates from various sources

Field Isolates	Arthrospore Isolates				
	1	2	3	4	5
1141C	P	P	P	P	P
1354B	P	P	P	P	P
1516	P	P	P	P	P
2002	P	P	P	P	P
2250	M	M	M	M	M
2261	M	M	M	M	M
LB5S	I	I	I	I	I
LB5W	M	M	M	M	M
LB2	M	M	M	M	M
LB3	M	M	M	M	M

I : Isolates intermingle with no obvious interaction
M : Line of demarcation consisting of mounded mycelium
P : Isolates overlap slightly; no mounds; but agar moderately pigmented under overlapping mycelia

The first four isolates come from S.E. Queensland; the second four come from N. Queensland and the last two are from Malaysia

The results of pairings of field isolates suggest that nearly all isolates came from different clones (Table 7.10). All but four of the Queensland isolates came from different plantation estates at least 20 km apart. Two isolates (1141C and 1354B) came from adjacent plantation compartments on the one estate, and the collection sites were approximately 400 m apart. Mycelia of these two isolates intermingled freely and thus were probably from the same "clone". The other two isolates (LB5S and LB5W) came from the one hoop pine stem, one from a basidiocarp and the other from the wood immediately behind the basidiocarp. The obvious interaction between mycelia of these two isolates suggests they differ genetically.

Table 7.10 Interactions of pigmented field isolates paired in all possible combinations

Isolate Number	1141C	1354B	1516	2002	2250	2261	LB5S	LB5W	LB2	LB3
1141C	I	I	P	M	M	P	M	M	M	M
1354B	I	I	P	M	M	M	M	M	M	M
1516	P	P	I	M	P	M	P	P	M	M
2002	M	M	M	I	M	M	M	M	M	M
2250	M	M	P	M	I	M	M	M	M	M
2261	P	M	M	M	M	I	M	M	M	M
LB5S	M	M	P	M	M	M	I	M	M	M
LB5W	M	M	P	M	M	M	M	I	M	M
LB2	M	M	M	M	M	M	M	M	I	M
LB3	M	M	M	M	M	M	M	M	M	I

I : Isolates intermingle with no obvious interaction
M : Line of demarcation consisting of mounded mycelium
P : Isolates overlap slightly; no mounds; but agar variously pigmented under overlapping mycelia

The first four isolates come from S.E. Queensland; the second four come from N. Queensland and the last two are from Malaysia

The significance of the results of pairings of unpigmented variants with each other and with normal pigmented isolates (Table 7.11) is obscure. The patterns of interactions varied among the variants, and none intermingled with the pigmented isolate of origin. Two variants (2250U and LB3U) intermingled with other pigmented isolates, even of different geographical origin.

Table 7.11 Interactions of unpigmented variants and field isolates

Isolate Number	1516U	2250U	LB3U	1141C	1354B	1516	2002	2250	2261	LB5S	LB5W	LB2	LB3
1516U	I	M	M	M	M	P	M	M	P	M	M	M	M
2250U	M	I	M	I	M	I	I	P	I	M	M	M	M
LB3U	M	M	I	M	M	I	M	P	M	M	P	P	M

I : Isolates intermingle with no obvious interaction

M : Line of demarcation consisting of mounded mycelium

P : Isolates overlap slightly; no mounds; but agar variously pigmented under overlapping mycelia

7.4 DISCUSSION

In studies on sexual incompatibility systems in other Hymenomycetes, workers have investigated species with haploid nuclei in basidiospores, or they apparently assumed the nuclei were haploid. The studies on the nuclear life history of *P. noxius* reported in Chapter 6 showed that basidiospores contained 0-3 nuclei and that nuclei could be haploid, diploid or perhaps triploid (Sections 6.2.3.3 and 6.4.3.2). Data in Figure 6.10 (Section 6.4.3.2) suggest that the following nuclear conditions prevailed among 95% of nucleated basidiospores: (i) uninucleate haploid, (ii) uninucleate diploid and (iii) binucleate haploid. The nuclei in the

binucleate spores probably differ genetically but do not have compatible mating factors: Data in Figure 6.10 show that the dimorphic cultures originating from some basidiospores (Section 3.2.2.3) contain only haploid nuclei. It appears reasonable to assume that these cultures originated from binucleate spores, and because sectors arising in these cultures were of two distinct morphological types, that the two nuclei are genetically different. However, as the cultures did not resemble the context isolate from the same basidiocarp, the nuclei probably do not have compatible mating factors. Thus, binucleate spores appear to have nuclei with common A or common B mating factors. The "intermediate" monobasidiospore cultures (Section 3.2.2.3) were more stable, and contained nuclei which were predominantly diploid (Figure 6.10), and thus they appear to have originated from spores with diploid nuclei. Stable diploid nuclei of all four possible combinations of mating factors have been grown in homokaryotic mycelia of some Hymenomycetes: A=B= and A≠B= diploids have been isolated from *Schizophyllum commune* (Parag & Nachman, 1966; Mills & Ellingboe, 1969) and A=B≠ diploids, from *Coprinus lagopus* (Casselton, 1965); and the normal vegetative mycelia of *Armillariella mellea* is a A≠B≠ diploid homokaryon (Peabody *et al.*, 1978; Ullrich & Anderson, 1978).

The complexity of the interactions between monobasidiospore isolates of *P. noxius* may be better appreciated in the light of studies by Casselton (1965) and Casselton and Lewis (1966) on *C. lagopus*. In those studies, a synthesized common-A diploid homokaryon of the fungus was paired with haploid homokaryons, haploid dikaryons and other diploid homokaryons. Thus, there appear to be some parallels between their studies and the situation which appears to prevail in *P. noxius*. Results of these studies which seem relevant to the present study are: (i) the

diploid homokaryon was very stable but whilst haploid segregants were rare, aneuploid intermediates were identified. (ii) The diploid homokaryon was dikaryotized in all pairings and thus the presence of common A or common B factors did not prevent the formation of a dikaryon; but the rate of dikaryotization was much slower than that which occurred when compatible homokaryons were paired. (iii) The diploid nucleus became unstable when a haploid nucleus was introduced into the mycelium and it haploidized via aneuploidy. However, the diploid nucleus often persisted to karyogamy in the basidium and a triploid fusion nucleus was formed. (iv) Dikaryotization by another diploid mycelium was very slow and was not complete; only small sectors of the dikaryon occurred in cultures. Either or both nuclei haploidize, and whilst both nuclei could persist to karyogamy in the case of a diploid-haploid cross, in no case did both nuclei persist in diploid-diploid crosses. (v) If haploidization of the diploid nucleus was completed before karyogamy, some chromosomes were not present in the basidiospore progeny, but if it had not commenced before karyogamy, the nuclear population among basidiospores was heterogeneous. If these phenomena also occur in *P. noxius*, they could explain the many different "patterns of interaction" found among basidiospore isolates. The slow rate of dikaryotization of diploid mycelia might also explain the failure to recognize compatible pairings in *P. noxius*.

Somatic mycelia of *P. noxius* contain haploid to octaploid (but predominantly diploid) nuclei; mycelia of the "intermediate" cultural type of monobasidiospore isolates contain haploid to tetraploid (but predominantly diploid) nuclei, and the average ploidy level of nuclei is less than that in somatic mycelia (Section 6.4.3.2). Casselton (1965) and Casselton and Lewis (1966) did not discuss the possibility of ploidy

levels above diploid in dikaryotized mycelia of *C. lagopus*. Hence, the mechanism for converting the nuclear condition of monobasidiospore mycelia to that existing in somatic mycelia through "compatible" pairings remains obscure. Perhaps nothing more is required than the disruption of the stability of the diploid nucleus in a monosporous mycelium by an introduced nucleus to initiate the mechanism previously proposed (Section 6.5) to explain the nuclear condition in somatic mycelia.

Studies on sexual incompatibility in hymenomycete species which lack clamp connections on somatic hyphae are rare. The present studies and those on two other clampless species, *Polyporus schweinitzii* (Barrett & Uscuplic, 1971) and *Phellinus weirii* (Hansen, 1979b) indicate that considerable difficulties can occur in interpreting interactions recorded. Hansen (1979b) suggested from his observations that a different system of incompatibility might operate in *P. weirii*. Observations in the present studies on variation in *P. noxius* have revealed the possibility of another system of incompatibility, but one which is basically tetrapolar. This system might also operate in *P. weirii*, and this could be investigated by examining the ploidy of nuclei at all phases of the life cycle of that fungus.

The occurrence of intersterile groups apparent among isolates of *P. noxius* has been reported for a number of Hymenomycetes (Burnett, 1976). It has also been recently reported in the important root pathogens *A. mellea* and *Heterobasidion annosum*: Korhonen (1978b) identified at least three intersterile groups among isolates of *H. annosum* from worldwide sources, and two occurred in Finland; Ullrich and Anderson (1978) found six such groups among isolates of *A. mellea* from the northeast of the United States.

The studies on interfertility and vegetative incompatibility indicated

that *P. noxius* in Malaysia, north Queensland and southeastern Queensland have separated genetically, and also that genetically different "clones" have arisen within each geographical locality.

Naturally occurring "clones" of pathogenic Hymenomycetes may vary considerably in size. Remarkably large "clones" of *A. mellea* covering at least 600 ha have been found in stands of *Pinus ponderosa* Laws. (Shaw & Roth, 1976), but diameters of "clones" of the fungus are usually 10-150 m (Korhonen, 1978a; Ullrich & Anderson, 1978). On the other hand, genetically different strains of *P. schweinitzii* have been isolated from adjacent trees in plantations of *Picea sitchensis* (Bong.) Carr. (Barrett & Uscuplic, 1971). In the present studies, genetically similar isolates of *P. noxius* were collected 400 m apart in young (<5 years) hoop pine plantations in southeastern Queensland. Continuity of the clone over this large area suggests that the distribution of the fungus was by vegetative growth rather than by spores. This is possible as the original vegetation on the area was rainforest, and Corner (1932) and Thrower (1965) have observed that basidiocarps rarely, if ever, develop deep in the rainforest; this is supported by personal observations of the author. In contrast, two genetically different isolates of *P. noxius* were obtained from the same hoop pine stem in north Queensland. This suggests that infection came from two sources, probably through vegetative growth via the roots, and by spores; basidiocarps of the fungus were common on dead trees in this plantation. The distribution of "clones" of the fungus in hoop pine plantations obviously warrants further investigation.

CHAPTER 8

GENERAL CONCLUSION

Control of root-rot induced by *P. noxius* is desirable in plantations of hoop pine in Queensland, and some appreciation of the variability of the pathogen was considered useful in assessing applicability of options for control (Chapter 1); hence, the rationale for the present project. The present studies are apparently the first detailed investigations into variation in *P. noxius*. Thus, they were essentially exploratory in nature.

Studies on field isolates, supported by parallel studies on monobasidiospore isolates from a single fructification, provided evidence of genetic flexibility in *P. noxius*. Cultures of such isolates were very variable in appearance and morphology (Chapter 3), and they frequently gave rise spontaneously to pigmented or unpigmented sectors which differed markedly in growth rate and in a number of physiological traits (Chapter 4), and in pathogenicity (Chapter 5). Further, a number of nuclear mechanisms (e.g. heterokaryosis, extensive nuclear migration and the occurrence of apparently unstable nuclei with a heterogeneity of ploidy levels - Chapters 6 and 7) which could account for much of the variability encountered in the fungus were recognized. The tropical rainforest in which *P. noxius* is widespread, is perhaps the most complex of habitats for fungi: usually, a very large number of species of higher plants is present on a very limited area, and the forest floor has an accumulation of decaying leaves, twigs, branches,bark and wood (Francis, 1951). Hence,

genetic flexibility in a facultative parasite such as *P. noxius* would be advantageous in accommodating the continual changes in substrate and environment with which it is presumably confronted.

The mechanism by which unpigmented sectors developed pigmentation after they overgrew the malt-extract agar substrate used in these studies is unknown. The phenomenon can be explained in terms of nuclear proportions in a heterokaryotic mycelium. Cells of hyphae in unpigmented sectors may contain nuclei which are predominantly or solely of a particular genetic constitution, e.g. nuclei with a chromosome carrying a recessive allelomorph 'p' (unpigmented). The unpigmented condition of young hyphae would presuppose that such nuclei divide or migrate to hyphal apices while nuclei with the dominant allelomorph 'P' (pigmented) do not migrate. Cytological observations made in the present studies indicated that such preferential division or migration may occur: apical cells at the advancing margin of cultures often contained fewer nuclei than their subapical cells. Pigmentation in these sectors might develop subsequently as a consequence of division and migration of the 'P' nuclei through the already established mycelium. Since the already formed cells would have established their dimensions (especially length), they would not accommodate many additional nuclei. Hence, when migration of the 'P' nuclei is complete, such cells probably would contain more 'p' than 'P' nuclei. Evidence for this hypothesis is quite good as these sectors never developed the intensity of pigmentation of the pigmented mycelia from which they arose.

Malaysian isolates grew faster, had lower pH optima, and were more virulent than Queensland isolates. In the relevant chapters (Chapters 4 and 5), suggestions were offered for studies which might give additional data on the variation in physiology and pathogenicity, within and among isolates of *P. noxius* from various sources. However, it is considered

that further studies on the nuclear mechanisms for variation in *P. noxius* should receive priority at this juncture.

A satisfactory technique for staining nuclei in the various elements of basidiocarps of *P. noxius* (e.g. the subhymenium, basidia and attached basidiospores) is essential for a more complete understanding of the nuclear life history of the fungus, e.g. determinations of the ploidy of nuclei in these elements will assist the elucidation of the origin of the heterogeneity of ploidy levels in somatic hyphae. The failure to develop such a technique in the present studies was attributed to a mucilaginous layer on the hymenium and agglutination of the subhymenial hyphae impeding the penetration of one or more of the chemicals used in the staining process. The mucilaginous layer and agglutination are included in descriptions of *P. noxius* basidiocarps by Corner (1932) and Cunningham (1965). Corner (1932) tested unsuccessfully a number of solvents on these substances: weak acids, chloroform, ether, xylene and absolute alcohol. Other suitable solvents (i.e. those which would not interfere with the chemistry of the staining process) should be sought and tested.

The system of incompatibility operating in *P. noxius* requires further study. Pairings of monobasidiospore isolates from additional basidiocarps are necessary; these isolates could be used also for further investigations on interfertility. However, the difficulties in interpreting interactions in the present studies (Chapter 7) still may be encountered. These difficulties might be overcome if genetically homogeneous cultures could be obtained; single arthrospores from monobasidiospore isolates (particularly those with appressed pigmented and unpigmented cultural types) might be a source for such cultures.

In the latter part of this project (Chapters 6 and 7), an understanding of the variability in *P. noxius* was sought through a study of nuclear

mechanisms operating in the fungus; other mechanisms, e.g. cytoplasmic variation, also may account for some of the variability. Cytoplasmic variation is thought to be responsible for much of the spontaneous variability in cultural behaviour and pathogenicity, and race differentiation in *Phytophthora infestans* (Mont.) d By. (Caten & Jinks, 1968; Leach & Rich, 1969; Caten, 1970). However, much of the information implying extra-nuclear inheritance has been carried out with strains with a poorly-defined genetical background; and until better-defined strains are available, and a more complete knowledge of nuclear cytology of pathogens such as *P. infestans* is attained, it is likely that difficulties in interpreting results on a Mendelian basis will continue to give rise to suggestions of extrachromosomal determinants (Webster, 1974). The importance of knowledge of the cytology of nuclear division as a source of variation in fungi is highlighted by the observations (Chapter 6) made in the present project.

The great apparently genotypic variability found in *P. noxius* might imply that certain genotypes of the fungus would be rampant pathogens. Observations by the author, however, suggest that the fungus induces mortality in its hosts in the virgin rainforest only occasionally. Two factors may keep the fungus in check in its natural habitat: host resistance and stabilizing selection. Examples of resistance phenomena operating in hosts of fungal root pathogens are given by Rishbeth (1972). Much of the morphological and physiological (and presumably pathogenic) variation observed in the laboratory is not detected in nature because of stabilizing selection (Burnett, 1976). The pressures of natural selection presumably eliminate many of the mutants which appear in the laboratory where such pressures are relaxed. For example, it is doubtful whether the tetrapolar unpigmented variant (mutant?) which arose from a

number of field isolates of *P. noxius* would survive under natural conditions: the variants appeared to have no conferred advantage over the other cultural types since most of their physiological traits were similar to those of the isolates from which they originated, but they grew more slowly and were non-pathogenic (Chapters 4 and 5). It has been suggested that the saprophytic substrate is also a stabilizing force for non-obligate fungal parasites (Nelson, 1972).

Knowledge of the role played by spores in the spread of the pathogen is necessary also for the assessment of the various methods of control. The importance of root contact in the local transmission of root-rot caused by *P. noxius* has been clearly established (Anon., 1967; Bolland, 1978). Successful basidiospore inoculations have been achieved on fresh stumps of rubber (Newsam, 1967) and hoop pine (Bolland, 1978), although only at low levels in the latter case (6% of inoculated stumps). Results of tests for interfertility (Section 7.3) suggest that basidiospores might be important also for the introduction of new genes into already established genotypes among somatic mycelia. The role of arthrospores requires elucidation. In the only study reported, arthrospore inoculations on hoop pine stumps were unsuccessful (Bolland, 1978). It is generally assumed that the function of oidia is the asexual propagation of a species, but Kemp (1975) has shown that in *Coprinus* spp. they can act as spermatia in dikaryotizing homokaryotic mycelia. Further, they may function as ecological barriers against hyphae of closely related species which invade the same environment. While fusion occurs between oidia of one species and mycelia of another, vacuolation and death of the heteroplasmon which forms occurs within 2 to 4 hours of the fusion. These findings should be considered in future investigations into the role of arthrospores in the life and disease cycles of *P. noxius*.

Control methods which have been used elsewhere against root-rot caused by *P. noxius* (e.g. in rubber plantations) are either too costly or logistically inappropriate for pine plantations (Chapter 1). Presumably it would be very difficult to restrict further local spread of the pathogen once it is established on a particular plantation site. Thus, control may be limited to protection of stumps of harvested trees against air-borne infection. The accounts of Rishbeth (1975, 1976) on chemical and biological protection of stumps in forestry plantations against the hymenomycete root pathogens *Armillariella mellea* and *Heterobasidion annosum* are useful references to consult should spores of *P. noxius* be found to play an important role in the spread of the pathogen in hoop pine plantations in Queensland.

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APPENDIX 1 (A1)

RECORDED WORLD DISTRIBUTION OF *PHELLINUS NOXIUS*

AFRICA : Cameroon, Congo, Ghana, Ivory Coast, Kenya, Nigeria, Tanzania, Uganda, Zaïre

ASIA : India, Indonesia, Malaysia, the Philippines, Singapore, Sri Lanka, Taiwan

AUSTRALASIA : Australia, Norfolk Island, Papua-New Guinea

PACIFIC ISLANDS : Fiji, New Hebrides, Samoa

CENTRAL AMERICA : Costa Rica

CARIBBEAN : Cuba, Peurto Rico

SOUTH AMERICA : Brazil

(References: Corner, 1932; Fidalgo, 1968; Anon., 1969; Peglar & Waterston, 1969; Anon., 1977; Dr B.N. Brown, pers. comm.)

See also Figure A1.1

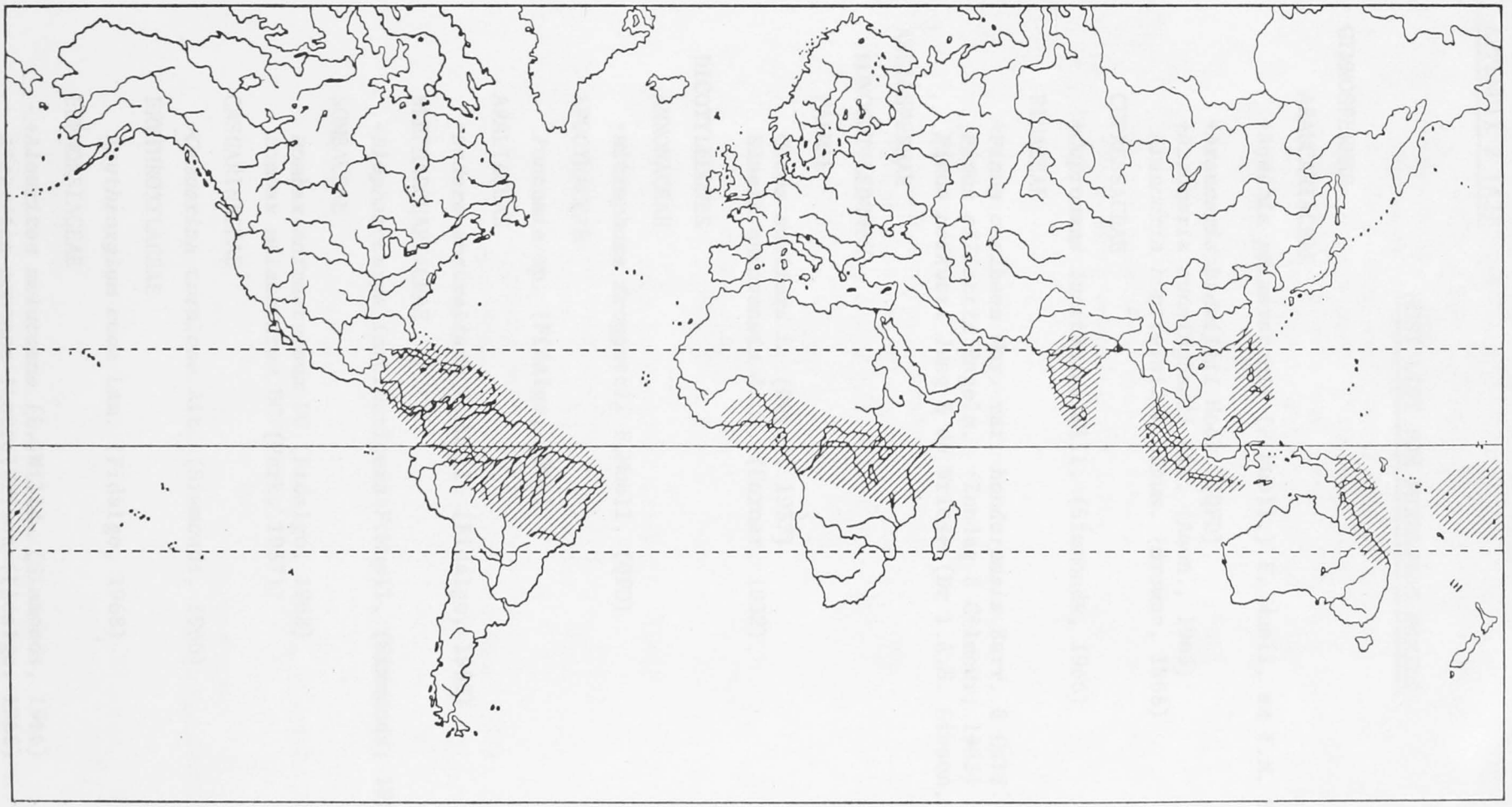


Figure A1.1. Recorded world distribution of *P. noxius*.

APPENDIX 2 (A2)

HOST LIST FOR *PHELLINUS NOXIUS*

GYMNOSPERMAE

ARAUCARIACEAE

+*Agathis palmerstonii* (F.Muell.) F. Muell. ex F.M. Bail. (Simmonds, 1966)

+*Araucaria bidwillii* Hook. (QFD)
+*Araucaria cunninghamii* Ait. (Anon., 1963)
Araucaria hunsteinii K.Schum. (Browne, 1968)

CUPRESSACEAE

+*Cupressus lusitanica* Mill. (Simmonds, 1966)

PINACEAE

+*Pinus caribaea* Mor. var. *hondurensis* Barr. & Gold. (QFD)
Pinus elliottii Engelm. (Zondag & Gilmour, 1963)
Pinus merkusii Jung & de Vriese (Dr I.A.S. Gibson, pers.comm.)

ANGIOSPERMAE

MONOCOTYLEDONES

PALMAE

Cocos nucifera L. (Dwyer, 1937)
Elaeis guineensis Jacq. (Corner, 1932)

DICOTYLEDONES

ANNONACEAE

+*Mitrephora froggattii* F.Muell. (QFD)

APOCYNACEAE

Funtumia sp. (Fidalgo, 1968)

ARALIACEAE

+*Hedera australiana* F.Muell. (Fidalgo, 1968)

BLEPHAROCARYACEAE

+*Blepharocarya involucrigera* F.Muell. (Simmonds, 1966)

BOMBACEAE

Bombax anfractuosum DC (Fidalgo, 1968)
Bombax malabaricum DC (Park, 1937)

CASUARINACEAE

+*Casuarina torulosa* Ait. (Simmonds, 1966)

ERYTHROXYLACEAE

Erythroxylum coca Lam. (Fidalgo, 1968)

EUPHORBIACEAE

+*Aleurites moluccana* (L.)Willd. (Simmonds, 1966)
Aleurites montana (Lour.)Wilson (Fidalgo, 1968)

APPENDIX 2 (A2) cont.

+*Clutia* sp. (QFD)
Codiaeum variegatum Blume (Fidalgo, 1968)
Hevea brasiliensis Muell.Arg. (Corner, 1932)
Hura crepitans L. (Fidalgo, 1968)
Manihot glaziovii Muell.Arg. (Fidalgo, 1968)
Manihot utilissima Pohl. (Fidalgo, 1968)

FLACOURTIACEAE

Hydnocarpus kurzii King (Fidalgo, 1968)

FLINDERSIACEAE

+*Flindersia brayleyana* F.Muell. (Simmonds, 1966)
+*Flindersia pimentaliana* F.Muell. (QFD)
+*Flindersia schottiana* F.Muell. (QFD)

GUTTIFERAE

Garcinia mangostana L. (Fidalgo, 1968)

LAURACEAE

Cinnamomum sp. (Fidalgo, 1968)
+*Cryptocarya mackinnoniana* F.Muell. (QFD)
Persea americana Mill (Fidalgo, 1968)

LEGUMINOSAE

Acacia sp. (Fidalgo, 1968)
+*Acacia aulacocarpa* A.Cunn. ex Benth. (Fidalgo, 1968)
Albizia falcata (L.) Back. (Steyaert, 1948)
Albizia lebbek (L.) Benth. (Steyaert, 1948)
Bauhinia sp. (Sawada, 1935)
Brownea grandiceps Jacq. (Fidalgo, 1968)
Cassia siamea Lam. (Fidalgo, 1968)
Crotolaria anagyroides HBK (Fidalgo, 1968)
+*Delonix regia* (Boj.)Raf. (Simmonds, 1966)
Erythrina sp. (Fidalgo, 1968)
Leucaena glauca Benth. (Fidalgo, 1968)
Pterocarpus indicus Willd. (Van der Goot, 1934)
Tephrosia vogelii Hook. (Fidalgo, 1968)

MALVACEAE

Thespesia populnea Soland & Correa (Fidalgo, 1968)

MELIACEAE

Cedrella mexicana M.J. Roem. (Zondag & Gilmour, 1963)
Khaya ivorensis A. (Fidalgo, 1968)
+*Khaya nyassica* Stapf. ex Bak (QFD)
Swietenia macrophylla King (Van der Goot, 1934)
Swietenia mahogoni Jacq. (Van der Goot, 1934)

MORACEAE

Artocarpus blumei Trec. (S'Jacob & DeFluiter, 1938)
Artocarpus integra (Thumb.)Merr. (Fidalgo, 1968)
Artocarpus integrifolia L. (Fidalgo, 1968)
Castilla elastica Cerv. (Fidalgo, 1968)
Chlorophora excelsa Benth. & Hook. (Fidalgo, 1968)
+*Ficus septica* Burm.f. (QFD)
+*Malaisia scandens* (Lour.)Planch. (QFD)

APPENDIX 2 (A2) cont.

MYRISTICACEAE

Myristica fragrans Houtt. (Leefmans, 1933)

MYRTACEAE

+*Eucalyptus drepanophylla* F.Muell ex Benth. (Simmonds, 1966)
+*Eucalyptus citriodora* Hook. (QFD)

PROTEACEAE

+*Grevillea robusta* A. Cunn. ex R.Br. (Simmonds, 1966)
+*Stenocarpus sinuatus* Endl. (Simmonds, 1966)

ROSACEAE

+*Pygeum turneranum* F.M.Bail. (QFD)
+*Rosa* spp. (QFD)

RUBIACEAE

Cinchona spp. (Keuchenius, 1939)
Coffea arabica L. (Fidalgo, 1968)

RUTACEAE

Citrus noblis Lour. (Fidalgo, 1968)

SAPINDACEAE

+*Castanospora alphandii* F.Muell. (QFD)
Nephelium lappaceum L. (Fidalgo, 1968)

SOLANACEAE

Brunfelsia americana L. (Fidalgo, 1968)
Capsicum sp. (Turner, 1968)

STERCULIACEAE

Cola nitida (Vent.)A.Chevalier (Fidalgo, 1968)
+*Heritiera* spp. (QFD)
Theobroma cacao L. (Fidalgo, 1968)

THEACEAE

Camellia sinensis L. (Corner, 1932)

ULMACEAE

Celtis sp. (Fidalgo, 1968)
+*Trema orientalis* Blume (QFD)

UTICACEAE

Boehmeria nivea(L.) Gaudich. (Fidalgo, 1968)

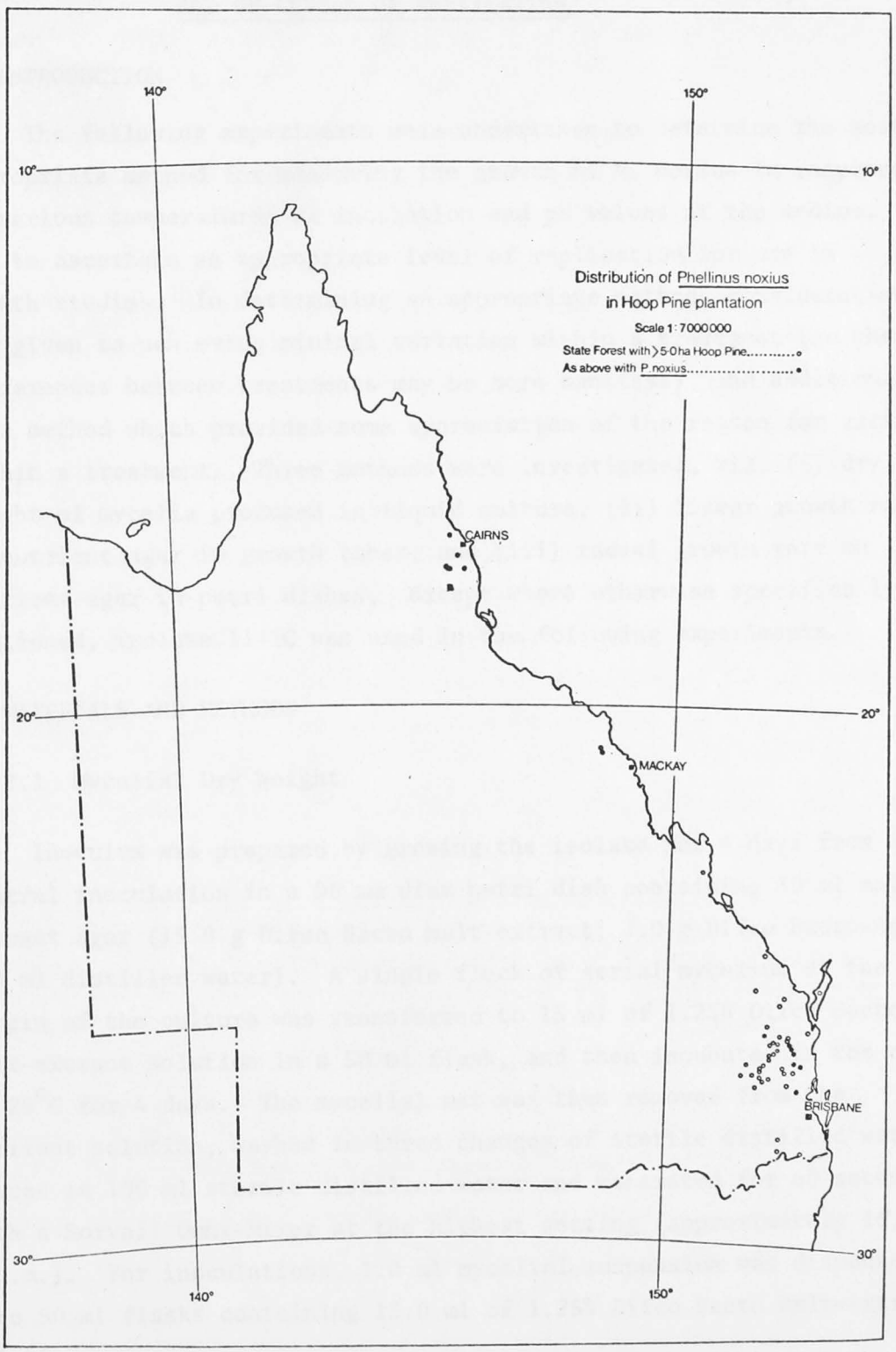
VERBENACEAE

Duranta repens L. (Fidalgo, 1968)
+*Lantana camara* L. (QFD)
Tectona grandis L. (Fidalgo, 1968)
Vitex altissima L. (Fidalgo, 1968)

The nomenclature and classification of Willis (1973) was followed in the above compilation.
+ : Species recorded as hosts in Queensland, Australia.
QFD : Unpublished accessions of the Department of Forestry, Queensland.

APPENDIX 3 (A3)

RECORDED DISTRIBUTION OF *P. NOXIUS* IN PLANTATIONS OF *ARAUCARIA CUNNINGHAMII* IN QUEENSLAND



APPENDIX 4 (A4)

ASSESSMENTS OF METHODS FOR MEASURING GROWTH AND OF LEVELS OF REPLICATION

1. INTRODUCTION

The following experiments were undertaken to determine the most appropriate method for measuring the growth of *P. noxius* in response to various temperatures of incubation and pH values of the medium, and to ascertain an appropriate level of replication for use in growth studies. In determining an appropriate method, consideration was given to achieving minimal variation within a treatment (so that differences between treatments may be more manifest), and additionally to a method which provided some appreciation of the reason for variation within a treatment. Three methods were investigated, viz. (i) dry weight of mycelia produced in liquid culture, (ii) linear growth rate on nutrient agar in growth tubes, and (iii) radial growth rate on nutrient agar in petri dishes. Except where otherwise specifically mentioned, isolate 1141C was used in the following experiments.

2. MATERIALS AND METHODS

2.1 Mycelial Dry Weight

Inoculum was prepared by growing the isolate for 4 days from a central inoculation in a 90 mm diam petri dish containing 30 ml malt-extract agar (15.0 g Difco Bacto malt-extract; 2.0 g Difco Bacto-Agar; 100 ml distilled water). A single fleck of aerial mycelium at the margin of the culture was transferred to 15 ml of 1.25% Difco Bacto malt-extract solution in a 50 ml flask, and then incubated in the dark at 25^{o}C for 4 days. The mycelial mat was then removed from the nutrient solution, washed in three changes of sterile distilled water, placed in 100 ml sterile distilled water and macerated for 60 seconds with a Sorvall Omni-Mixer at the highest setting (approximately 16,000 r.p.m.). For inoculations, 1.0 ml mycelial suspension was dispensed into 50 ml flasks containing 15.0 ml of 1.25% Difco Bacto malt-extract solution.

Before comparisons could be made with other methods, preliminary

APPENDIX 4 (A4) cont.

studies were necessary to determine (i) the extent of shaking required to give the least variable results, and (ii) an appropriate period for incubation.

To determine the shaking required, four inoculated flasks were kept stationary, or shaken by hand for 20 seconds daily, or shaken continuously in an orbital incubator. All cultures were incubated in the dark at 25°C for 14 days. The mycelium from each flask was harvested on filter paper which had been previously dried to constant weight, washed with 50 ml distilled water, and dried to constant weight at 80°C for 3 days. Weights were recorded to the nearest 0.1 mg. Results are presented in Table A4.1. Continuously shaken cultures produced the greatest weight of dry mycelium, and still cultures, the least. However, the coefficient of variation was least for cultures shaken for 20 seconds daily. The larger variation among continuously shaken cultures was probably due in part to the adherence of some mycelia to the sides of each flask, and that among still cultures was probably due to poor aeration and nutrient status of the medium around individual hyphae. Hence, for dry weight determinations, flasks were shaken 20 seconds daily.

Table A4.1 Comparisons of dry weights (mg) of mycelia produced after 14 days in still and shaken liquid cultures

Flasks stationary	Flasks shaken 20 seconds daily	Flasks shaken continuously
13.9±3.2 a (23.0%)	17.4±1.1 a (6.3%)	26.9±4.6 b (17.1%)

Values are means of four replicates, ± standard deviations
Values followed by the same letter do not differ significantly at $p = 0.05$
Values in brackets are coefficients of variation = $\frac{\text{standard deviation}}{\text{mean}} \times 100\%$

An appropriate period for incubation of liquid cultures was determined by incubating four inoculated flasks in the dark at 25°C for 3, 6, 9, 12, 15, 18 or 21 days. Results are presented in Table A4.2. Mycelial dry

APPENDIX 4 (A4) cont.

weight increased with time for 15 days and then began to decline, but differences between results at 12, 15 and 18 days were not significant. Therefore, 15 days was selected as an appropriate period for incubation of liquid cultures.

Table A4.2 Dry weight (mg) of mycelia after various periods of incubation in liquid cultures shaken 20 seconds daily

Period of incubation (days)						
3	6	9	12	15	18	21
3.7 a	8.0 b	15.3cf	16.6d	16.8d	16.5def	15.9ef

Values followed by the same letter do not differ significantly at p=0.05. Values underlined are the maxima and do not differ significantly.

2.2. Rate of Linear Growth in Tubes

An inoculum culture was prepared by growing the isolate for 4 days from a central inoculation in a 90 mm diam petri dish containing 15 ml malt-extract agar (MEA: 12.5 g Difco Bacto malt-extract; 20.0 g Difco Bacto-Agar; 1000 ml distilled water). Fifteen ml MEA were poured into 38.0 cm x 1.5 cm (internal diam) glass growth tubes. One 4 mm diam plug was cut from the advancing margin of the inoculum culture and placed mycelium down on the surface of the medium at one end of each tube. Cultures were then incubated in the dark at 25°C. Ten days after inoculation, a line corresponding to the position of the margin of the culture was marked on each tube. Fifty days later, the linear growth of each culture from day 10 was measured and converted to growth rate (mm/24 hr). Results are given in Section 3.

2.3 Rate of Radial Growth in Petri Dishes

Growth of fungi in culture apparently follows a pattern. Definite phases can be recognized in the early development of cultures: (i) a phase of no growth, (ii) a phase of accelerating growth and (iii) a phase of linear growth (Lilly & Barnett, 1951). It seems necessary, then,

APPENDIX 4 (A4) cont.

to assess growth rates only during the phase of linear growth if other factors which may introduce variation (e.g., growth rates during the phase of accelerating growth) are to be avoided. Hence, preliminary studies were undertaken to determine the time after first growth is observed in cultures of *P. noxius* to the commencement of the linear phase of growth. This was determined for two isolates, 1141C and LB3 (an isolate with very rapid growth), and, because the commencement of the linear phase of growth might vary with the temperature of incubation, at three incubation temperatures.

Inoculum cultures were prepared, and growth rates were assessed, in 90 mm diam petri dishes containing 30 ml malt-extract agar (MEA: 12.5 g Difco Bacto malt-extract; 20.0 g Difco Bacto-Agar; 1000 ml distilled water). Inoculum cultures were grown for 4 days from central inoculations. One 4 mm diam plug was cut from the advancing margin of an inoculum culture and placed mycelium down at the edge of freshly prepared dishes of MEA. Eight dishes were inoculated per isolate, per temperature treatment. Cultures were incubated in the dark at 20°C, 25°C and 30°C.

Every 24 hr after inoculation, a line corresponding to the position of the margin of the culture was scribed on the reverse of each dish. The procedure was continued until the fastest growing culture of each isolate had overgrown the dish. Measurements were taken of radii of cultures after each 24 hr incubation. Three radii were measured for each culture: One along the diameter of the petri dish and two at 30° on either side of that radius. Results are presented in Fig. A4.1. Each point on a graph represents the mean of 24 measurements (eight dishes x three radii). The linear phase of growth of *P. noxius* commenced 48 hr after first growth was observed, irrespective of the isolate or the temperature of incubation. Hence, in following studies, growth rates were assessed along radii from the line scribed on the underside of the dish on the third day of observable growth.

2.4. Replication

An appropriate level of replication for use in growth studies on *P. noxius* was determined by inoculating eight replicates of either flasks,

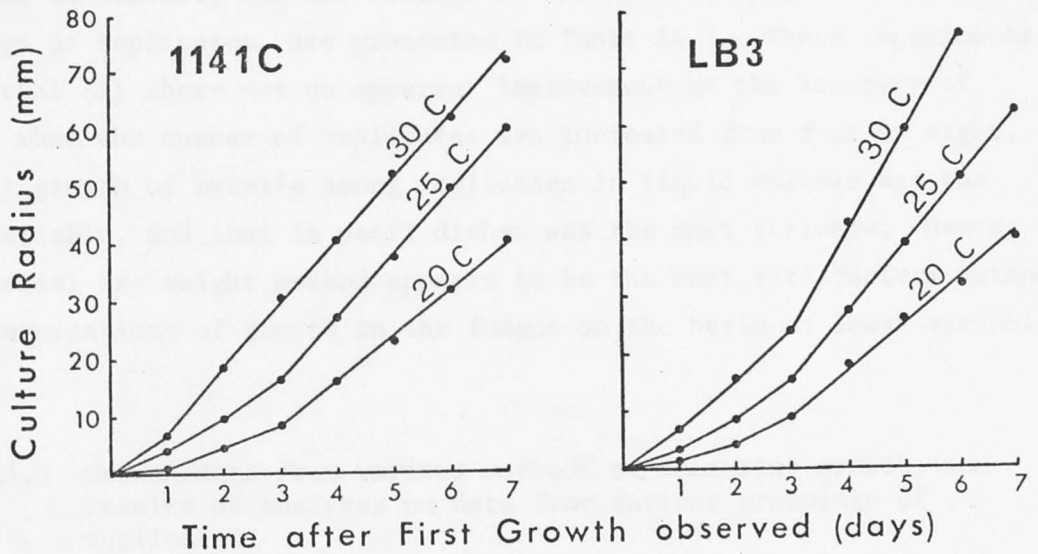


Figure A4.1. Radial growth of cultures of two isolates in petri dishes at three temperatures of incubation.

APPENDIX 4 (A4) cont.

growth tubes or dishes with isolate 1141C, and incubating the cultures in the dark at 25°C. Prior to incubation, each flask, tube or dish was assigned a different number from 1 to 8. At the completion of each experiment, means and standard deviations were calculated for the following groupings of replicates: Replicate numbers 1-4, 5-8 and 1-8. Comparisons were then made between the results of the different groupings.

3. RESULTS AND DISCUSSION

Growth recorded in the three methods tested for assessing growth of *P. noxius* in culture, and the results of analyses on data for the various groupings of replicates, are presented in Table A4.3. These experiments showed that (i) there was no apparent improvement in the accuracy of results when the number of replicates was increased from four to eight, and (ii) growth of mycelia among replicates in liquid culture was the least variable, and that in petri dishes was the most variable. Hence, the mycelial dry weight method appears to be the most satisfactory method for determinations of growth in the fungus on the basis of least variable results.

Table A4.3 Growth data from various methods of measuring growth, and results of analyses on data from various groupings of replicates

Method of Measuring Growth	Groupings of Replicates (Replicate Numbers)		
	1-4	4-8	1-8
Dry weight of mycelia in liquid culture (mg)	16.4±0.8 a (4.9%)	16.6±0.7 a (4.2%)	16.5±0.7 a (4.2%)
Linear growth rate in growth tubes (mm/24 hr)	4.8±0.5 b (10.4%)	4.3±0.4 b (9.3%)	4.5±0.5 b (11.1%)
Radial growth rate in petri dishes (mm/24 hr)	10.2±2.5 c (24.5%)	9.6±1.8 c (18.8%)	9.9±2.5 c (25.3%)

Values are means ± standard deviation.
Values followed by the same letter do not differ significantly at p=0.05.
Values in brackets are coefficients of variation (see below Table A4.1 for definition).

However, sectoring was observed in petri dishes. Cultures gave rise to two distinct cultural types: unpigmented mycelia and pigmented mycelia.

APPENDIX 4 (A4) cont.

The former mycelia grew faster than the latter. Since the present project is concerned with investigating variation in *P. noxius*, the study of growth in petri dishes might reveal differences between the two cultural types in other physiological traits, and so might give a better appreciation of the variation within and between isolates. This method was therefore selected for the growth studies in the present project.

APPENDIX 5 (A5)

GROWTH PATTERNS AND VARIABILITY IN RESULTS FOR GROWTH RATE WHEN INOCULA OF MYCELIAL FLECKS WERE USED IN STUDIES ON pH-GROWTH RELATIONSHIPS

1. INTRODUCTION

In the experiments reported in Appendix 4, growth tubes and petri dishes were inoculated with 4 mm diam plugs of malt-extract agar carrying mycelia of *P. noxius*. If such inocula were used to examine the effect of pH on the growth of the fungus, initial growth at adverse pH values would probably be supported by the inoculum substrate. Hence, it was decided to test the use of mycelial flecks free of supporting substrate as inocula for studies on the pH-growth relationships of the fungus. In the following experiment, the growth patterns and the accuracy of results for growth rate were determined using mycelial flecks on malt-extract agar at two pH values.

2. MATERIALS AND METHODS

Inoculum cultures of isolates 1141C and LB3 were prepared by growing each isolate for 4 days from a central inoculation in a petri dish containing 15% malt-extract agar. Malt-extract agar (30 ml) at pH 4.0 or 5.7 were dispensed into petri dishes 90 mm diam; the medium contained 12.5 g Difco Bacto malt-extract, 20.0 g Difco Bacto-Agar, 900 ml distilled water, and 100 ml phosphate buffer solution. Dishes were inoculated at the edge with a single fleck of aerial mycelium taken from the margin of an inoculum culture. Eight dishes were inoculated per isolate, per pH value, and were incubated in the dark at 25^{o}C.

Using the procedures described in Section 2.3 of Appendix 4, radii of cultures after each 24 hr incubation were determined for both isolates, and radial growth rate was determined for each culture of isolate 1141C. The means and standard deviations of radial growth rate were calculated for various groupings of replicates of isolate 1141C, as described in Section 2.4. of Appendix 4.

APPENDIX 5 (A5) cont.

3. RESULTS AND DISCUSSION

The radial growth of isolates with time are presented in Fig.A5.1. The patterns of growth were identical to those recorded in the earlier study with disc inocula (Section 2.3, Appendix 4): Phase of accelerating growth was followed by a phase of linear growth which commenced on the third day of observable growth irrespective of the isolate studied or the pH of the substrate.

Accuracy of data on growth rate was not improved by an increase in the number of replicates from four to eight (Table A5.1). Growth rates at pH 4.0 were more variable than those at 5.7, and this was probably due largely to the occurrence of the sectoring reported earlier (Section 3, Appendix 4) in a number of cultures on that medium.

Table A5.1 Growth rate (mm/24 hr) of isolate 1141C at two pH values, and results of analyses on data from various groupings of replicates

pH	Groupings of Replicates (Replicate Numbers)		
	1 - 4	4 - 8	1 - 8
4.0	7.0±2.3 a (33.3%)	6.4±1.8 a (27.5%)	6.7±2.0 a (30.7%)
5.7	8.9±1.6 b (18.0%)	9.3±2.5 b (26.9%)	9.1±2.3 b (25.3%)

Values are means ± standard deviations.
Values followed by the same letter do not differ significantly at p=0.05.
Values in brackets are coefficients of variation (see below Table A4.1 for definition).

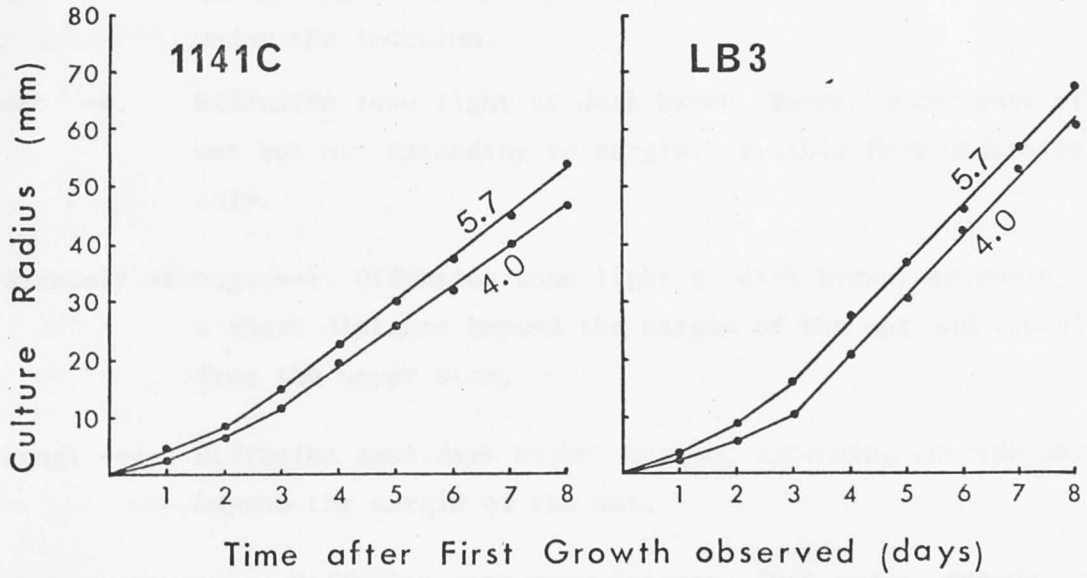


Figure A5.1. Radial growth of cultures of two isolates in petri dishes at pH 4.0 and 5.7.

APPENDIX 6 (A6)

DESCRIPTIONS OF THE STRENGTH OF REACTIONS FOR EXTRACELLULAR OXIDASE ON GAA AND TAA (FROM NOBLES,1948)

Negative: -. No brown discoloration of the agar under or about the mat.

Very weak: +. Diffusion zone light to dark brown, formed under inoculum at centre of mat and visible only from underside of dish. Where no growth takes place, a faint brown discoloration under the inoculum.

Weak: ++. Diffusion zone light to dark brown, formed under most of mat but not extending to margin. Visible from underside only.

Moderately strong: +++. Diffusion zone light to dark brown, extending a short distance beyond the margin of the mat and visible from the upper side.

Strong: ++++. Diffusion zone dark brown, opaque, extending considerably beyond the margin of the mat.

Very strong: +++++. Diffusion zone very intense, dark brown, opaque, forming a wide corona about the mat. Usually such intense reactions occur with species giving no growth on the medium, and are most common on gallic acid medium.

APPENDIX 7 (A7)

MEDIA FOR THE DETECTION OF EXTRACELLULAR ENZYMES
(FROM HANKIN AND ANAGNOSTAKIS, 1975, 1977)

All tests were made on prepoured plates. Test media above pH 6 were prepared in single-strength concentrations with agar included. Below pH 6 all media were prepared at double-strength concentrations without agar; the agar and basal medium were cooled separately to about 48°C, mixed together, and plates were poured immediately. All media contained sufficient substrate, other than the test substrate, to allow good growth of the fungus.

Pectolytic activity. The medium contained 500 ml mineral salts solution, 1 g yeast extract, 15 g agar, 5 g apple pectin and 500 ml distilled water; pH 7.0. The mineral salts solution contained per litre: 2 g $(NH_4)_2SO_4$; 4 g KH_2PO_4; 6 g Na_2HPO_4; 0.2 g $FeSO_4.7H_2O$; 1 mg $CaCl_2$; 10 µg $MnSO_4$; 70 µg $ZnSO_4$; 50 µg $CuSO_4$; 10 µg MoO_3; 10 µg H_3BO_3; pH 7 or 5 as required.

The medium at pH 7 was used to detect pectate transeliminase, and the same medium at pH 5 was used to detect pectin depolymerase.

After incubation plates were flooded with a 1% aqueous solution of hexadecyltrimethylammonium bromide. This reagent precipitates intact pectin in the medium and thus, clear zones around a colony in an otherwise opaque medium indicates degradation of pectin.

Amylolytic activity. The medium contained Difco Nutrient Agar plus 0.2% soluble starch, pH 6. After incubation, plates were flooded with an iodine solution and a yellow zone around a colony in an otherwise blue medium indicated amylolytic activity.

Lipolytic activity. Sorbitan monolaurate (Tween 20) was used as the lipid substrate. The medium contained per litre: 10 g Difco peptone; 5 g NaCl; 0.1 g $CaCl_2.2H_2O$; 20 g agar; pH 6. The Tween 20 was sterilized separately by autoclaving for 15 minutes at 121°C and 1 ml added per 100 ml of sterile and cooled basal medium.

APPENDIX 7 (A7) cont.

The formation of lipolytic enzymes by a colony was seen as either a visible precipitate due to the formation of crystals of the calcium salt of the lauric acid liberated by the enzyme, or as a clearing of such a precipitate around a colony due to the complete degradation of the salt of the fatty acid.

Proteolytic activity. The medium contained Difco Nutrient Agar plus 0.4% gelatin, pH 6. An 8% solution of gelatin in water was sterilized separately and added to the agar at the rate of 5 ml per 100 ml medium.

After incubation plates were flooded with an aqueous saturated solution of ammonium sulphate. If the enzyme was present, a clear zone was obvious around a colony, in the agar which was made more opaque by the precipitation of the protein by the reagent.

Phosphatase activity. An 0.01M aqueous solution of phenol-phthalein diphosphate (sodium salt) was prepared by gentle warming and sterilized by Millipore filtration. The solution (2 ml) was added to 98 ml of sterile, cooled Difco Plate Count Agar at pH 6.

After incubation, plates were inverted over a container of ammonium hydroxide. Colonies that turned pink to red were presumed to have degraded the substrate.

Deoxyribonuclease activity. The medium was Difco DNAase agar, pH 6.

After incubation, plates were flooded with 1 N HCl, and clear zones around colonies in an otherwise opaque medium indicated degradation of the deoxyribonucleic acid in the medium.

Ribonuclease activity. Torula yeast RNA was used as the test substrate. The medium contained per litre: 5 g glucose; 5 g Difco vitamin-free casamino acids; 5 g KH_2PO_4; 2 g Na Cl; 0.05 g $FeSO_4$; 0.5 g $MgSO_4.7H_20$; 15 g agar; pH 6. The Torula yeast RNA was prepared in water to give a concentration of 2 mg/ml of medium. It was dissolved by slow addition of 1 N NaOH and was added to the liquefied basal medium just prior to autoclaving. Plates were poured as soon as the medium had cooled to 50°C. After incubation, plates were flooded with 1 N HCl as with DNAase medium, and examined for clear zones around colonies in the opaque agar.

APPENDIX 7 (A7) cont.

Urease activity. A urea solution of 0.5 g per ml was prepared and sterilized by Millipore filtration. The solution (2 ml) was added to each 100 ml of previously sterilized and cooled Difco Plate Count Agar at pH 6, and plates were poured immediately.

After incubation, urease production was detected by pouring an overlay containing urea and an indicator over the surface of the plate. The overlay contained 100 ml 0.01 M phosphate buffer, pH 6; 1.5 g of agar; and 1 g urea. The urea was added after the liquefied agar-buffer mixture had cooled to 48°C to preclude hydrolysis. Before pouring, about 0.5 ml of a 1% solution of bromthymolblue was added. The colour of the overlay should be orange to yellow. If green, 0.1 N HCl was added to adjust the colour. Colonies producing urease turn the overlay blue. At least 2 hr was allowed before recording a negative result.

Cellulase activity. The medium contained 500 ml mineral salts solution (as given for the media testing pectolytic activity); 1 g yeast extract; 5 g carboxymethylcellulose (CMC); 10 g agar; 500 ml distilled water; pH 7.0.

After incubation, plates were flooded with 1% aqueous solution of hexadecyltrimethylammonium bromide. The reagent precipitates intact CMC in the medium leaving a clear zone where the CMC has been degraded.

APPENDIX 8 (A8)

VOLUMES OF $\frac{M}{3}$ SOLUTIONS OF PHOSPHATES ADDED PER 1000 ML OF MALT-EXTRACT AGAR TO GIVE DESIRED pH TREATMENTS (FROM CUMMINS, 1928)

Target pH	Ml of Phosphate Solution			
	H_3PO_4	NaH_2PO_4	Na_2HPO_4	Na_3PO_4
2.0	100			
3.0	32	68		
4.0	9	91		
5.0		99	1	
5.5		95	5	
6.0		83	17	
7.0		30	70	
8.0			93	7
9.0			78	22